Functional role of kainate receptors in the rat entorhinal cortex

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Functional role of kainate receptors in the rat entorhinal cortex.

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Abstract

An increasing amount of attention is being focussed on the physiological and pathological functions of kainate receptors (KAr), in mediating synaptic currents and presynaptically controlling transmitter release at excitatory and inhibitory synapses. The aims of this thesis were to determine the role of KAr in layer III of the rat entorhinal cortex (EC).

I have provided electrophysiological evidence for involvement of two types of KAr in excitatory glutamatergic neurotransmission in layer III: 1. A GluR5-containing presynaptic KAr, which facilitates glutamate release at these synapses and 2. A non-GluR5-containing postsynaptic KAr mediating synaptic currents. I have also demonstrated that several types of KAr act to increase synaptic inhibition in layer III. There appears to be a presynaptic facilitatory KAr on interneurone terminals, which acts to increase GABA release, and which may contain the GluR6 subunit. There is also a GluR5-containing KAr located on the soma/dendrites of interneurones, which drives GABA release, via direct excitation.

Slow wave oscillations (SWO) can be reliably generated in the EC by reducing $[Mg^{2+}]_o$. Cunningham *et al*. (2006b) have recently shown that GluR5 receptors are involved in the initiation and/or maintenance of SWO in the EC as they are blocked by UBP 302. I have shown that reducing $[Mg^{2+}]_o$ increases excitatory and inhibitory neurotransmission in layer III. A large part of the increased glutamate release was GluR5 KAr-dependent. Effects of GluR5 blockade suggest that the presynaptic GluR5 receptors on glutamatergic terminals become tonically activated in low Mg^{2+}. In addition, the increase in GABA release caused by reducing $[Mg^{2+}]_o$ is likely to be partly due to increased KAr activation of postsynaptic GluR5 receptors on interneurones. Layer V of the EC does not show pronounced SWO, and in preliminary experiments I have shown that KAr play a much less prominent role in glutamatergic transmission in this layer. Overall, my results provide good support for the suggestion that GluR5-containing KAr play an important role in SWO in layer III of the EC (Cunningham *et al*, 2006b).

Abbreviations

2-AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
aCSF	Artificial cerebro-spinal fluid
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAr	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AVOVA	Analysis of variance
ATPA	(RS)-2-Amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid
CGP 55845A	(2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydro xypropyl](phenylmethyl)phosphinic acid
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CSF	Cerebro-spinal fluid
DG	Dentate gyrus
EC	Entorhinal cortex
EEG	Electroencephalogram
eEPSC	Evoked excitatory postsynaptic current
eIPSC	Evoked inhibitory postsynaptic current
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
GABA	Gamma-aminobutyric acid
$GABA_A$r	Gamma-aminobutyric acid type A receptor
$GABA_B$r	Gamma-aminobutyric acid type B receptor
G-protein	GTP-binding protein
GYKI 53665	1-(4-aminophenyl)-3-methylcarbamoyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine
IEI	Inter-event interval
iGlur	Ionotropic glutamate receptor
i.m.	Intra-muscular
IPSC	Inhibitory postsynaptic current
KA	Kainic acid/kainate
KAr	Kainate receptor
KS	Kolmogorov-Smirnoff
LJP	Liquid junction potential

LPA1	Lysophosphatidic acid 1 receptor
mEPSC	Miniature excitatory postsynaptic current
$[Mg^{2+}]_o$	Extracellular magnesium concentration
mGlur	Metabotropic glutamate receptor
mIPSC	Miniature inhibitory postsynaptic current
MK 801	Dizocilpine maleate
MRI	Magnetic resonance imaging
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA	N-methyl-D-aspartic acid
NMDAr	N-methyl-D-aspartic acid receptor
PDC	L-trans-Pyrrolidine-2,4-dicarboxylic acid
PKA	Protein kinase A
PKA	Protein kinase C
PLC	Phospholipase C
PPD	Paired pulse depression
PPF	Paired pulse facilitation
PPR	Paired pulse ratio
QX-314	Lidocaine
R^2	Correlation co-efficient
sEPSC	Spontaneous excitatory postsynaptic current
sIPSC	Spontaneous inhibitory postsynaptic current
SR 95531	6-Imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide (gabazine)
SWO	Slow wave oscillations
SYM 2206	(±)-4-(4-Aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphthalazine
TA	Temporoammonic
TLE	Temporal lobe epilepsy
TTX	Tetrodotoxin
UBP 302	(S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrim idine-2,4-dione
VGCC	Voltage gated Ca^{2+} channels

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

This laboratory has been studying the role of ionotropic (iGlur) and metabotropic glutamate receptors (mGlur) in excitatory transmission and in the control of glutamate and GABA release in the entorhinal cortex (EC) for a number of years (e.g. Jones, 1987; 1994; Jones and Heinemann, 1989; Berretta and Jones, 1996a, b; Woodhall *et al.,* 2001a; Evans *et al.,* 2000; 2001). The EC is an essential structure in the temporal lobe, where it actively processes information entering and leaving the hippocampus, thus controlling the interactions between the hippocampus and the neocortex, and other areas of the limbic system (Witter *et al.,* 1989a; Delatour and Witter, 2002; Witter and Moser, 2006). The EC has been implicated in a number of neurological disorders including Alzheimer's disease, Parkinson's disease and schizophrenia (e.g. Van Hoesen *et al.,* 1991; Gomez-Isla *et al.,* 1996; Prasad *et al.,* 2004; Kövari *et al.,* 2003; Pennanen *et al.,* 2004). In addition it is also known to play a vital role in the initiation and propagation of temporal lobe epilepsy (TLE; see Jones and Woodhall, 2005; Tolner *et al.,* 2007).

Kainate receptors (KAr), like NMDA and AMPA receptors (NMDAr, AMPAr) are ligand-gated ion channels responsive to glutamate, the major excitatory neurotransmitter in the central nervous system (CNS). Postsynaptic KAr were initially found to mediate EPSCs much smaller and slower than AMPAr at hippocampal mossy fibre synapses (Castillo *et al.,* 1997; Vignes and Collingridge, 1997), and have since been found at various other synapses (Frerking *et al.,* 1998; DeVries and Schwartz, 1999; Kidd and Issac, 1999; Li *et al.,* 1999; Ali, 2003; Gryder and Rogawski, 2003). Presynaptic KAr exist at both excitatory (Agrawal and Evans, 1986; Frerking *et al.,* 2001; Schmitz *et al.,* 2001) and inhibitory synapses (Rodriguez-Moreno *et al.,* 1997; Cossart *et al.,* 2001) where, depending on the particular study, they act to increase or decrease transmitter release. Acute administration of kainate acid (KA) is frequently used to generate a chronic animal model of TLE, as it produces seizures and neuropathological changes closely reminiscent of those observed in patients (Ben-Ari, 1985). Epileptic activity induced by KA appears to arise initially in the EC before propagating to the hippocampus and other limbic areas (Ben-Ari *et al.,* 1981) indicating a role for KAr in the synchronisation leading to epileptogenesis. The chronic recurrent seizures that develop after a latent period are associated with substantial cell loss in the EC (Tolner *et al.,* 2005; 2007), although whether this is a

direct effect of KAr activation or as a consequence of hypersynchrony is still to be determined. What is becoming clear is that KAr are also important for other forms of network synchrony in the EC. Gamma frequency oscillations can be elicited in the EC by non-selective KAr agonists (Cunningham *et al.,* 2003; 2004). These were blocked by both AMPAr and $GABA_A$r antagonists suggesting AMPAr-mediated inputs to the GABAergic interneurones are involved in KAr driven rhythmogenesis. More recently it has been suggested that this gamma activity is largely dependent on GluR5-containing KAr (Stanger *et al.,* 2008). These KAr induced gamma oscillations have also been found to be markedly decreased in layer III in animal models of schizophrenia (Cunningham *et al.,* 2006a). Other oscillations may also depend on KAr in the EC. Slow wave oscillations (SWO), localised primarily to layer III, can be induced by a moderate reduction in extracellular magnesium ($[Mg^{2+}]_o$) and are abolished by a GluR5 selective KAr antagonist (Cunningham *et al.,* 2006b).

Thus, KAr seem to be involved in the generation of network rhythmicity in the EC at both physiological and pathological levels, but surprisingly little is known of the physiological roles that these receptors play at excitatory and inhibitory synapses in this area. A recent paper (West *et al.,* 2007) demonstrated a slow, KAr-mediated component of the glutamate EPSC in neurones in layer II and III of the rat EC, although it was more prominent in layer III. However, this is the only study of physiological role of KAr in the EC.

Thus, the aims of this thesis were to determine the role of KAr in layer III of the EC. In particular the work has:

- Characterised spontaneous inhibitory and excitatory synaptic transmission in layer III of the EC.
- Determined the role of presynaptic KAr in controlling spontaneous glutamate release at excitatory synapses.
- Examined the contribution of postsynaptic KAr at glutamate synapses.
- Defined the role of presynaptic KAr in the release of GABA in layer III.
- Determined how manipulations that elicit slow oscillatory activity may alter pre- and postsynaptic KAr function.

1.2. The entorhinal cortex

The EC is located in the temporal lobe and is a pivotal part of the limbic system. Due to its proximity to the hippocampus it has also been referred to as part of the parahippocampal region, together with the hippocampus itself, the DG (DG), the peri- and postrhinal cortices and the subiculum. The EC is uniquely positioned to serve as an interface between the neocortex and hippocampal formation and, as such, it acts as a gateway, actively pre-processing and selecting information directed afferent and efferent to the hippocampus. These structures are thought to be vital in memory and cognition (Zola Morgan and Squire, 1993; Myhrer, 1989). The cognitive and memory deficits seen in many dementias, including Alzheimer's disease, Parkinson's disease and Pick's disease, have been attributed to neuropathological changes in this region, particularly the EC (Braak and Braak, 1992; Braak *et al*., 2000). In addition, dysfunction of the EC is commonly associated with TLE. Atrophy of the EC has been seen both in animal models (Wozny *et al.,* 2005; Du *et al.,* 1995) and in patients (Bartolomei *et al.,* 2005) and removal of the EC is vital for control of TLE when surgical intervention is required due to pharmaco-resistance (Goldring *et al.,* 1992).

1.2.1. Anatomy of the EC

The area was first described by Cajal in 1901, although it was not termed the EC until 1909, when Brodmann referred to Area 28 as *area entorhinalis*. It resides in the medial temporal lobe, specifically within the rhinal sulcus in the rostral ventromedial surface. The rat brain does not contain a medial temporal lobe and the EC is located at the most caudal, ventral, and lateral aspect of the brain (Figure 1.1A). The EC curves around the rostral surface of the hippocampus as is indicated in Figure 1.1B. The EC is traditionally subdivided into the medial and lateral cortices, although more recently these have been subdivided further into six sub-regions (Insausti *et al.,* 1997). The medial and lateral divisions of the EC have strikingly different afferent and efferent connections (Kerr *et al.,* 2007) and are thought to have distinct information processing functions (Hargreaves *et al.,* 2005). This laboratory is principally concerned with the medial EC of the rat, which is where all of the experiments for this thesis have taken place.

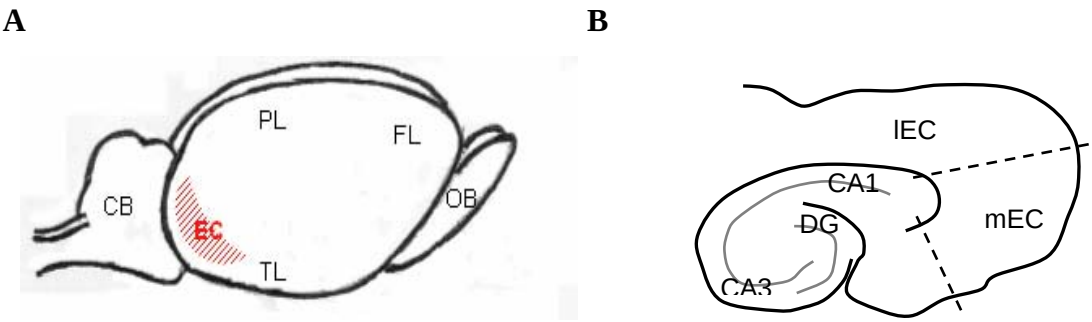


Figure 1.1. Schematic diagrams of the location of the EC. *A. Schematic diagram of a lateral view of the rat brain showing the position of the EC. Also identifies the cerebellum (CB), parietal lobe (PL), temporal lobe (TL), frontal lobe (FL) and the olfactory bulb (OB). B. Schematic diagram of an EC-hippocampal slice indicating the position of the medial and lateral EC in relation to the CA1 and CA3 regions of the hippocampus and the dentate gyrus (DG).*

1.2.2. Intrinsic connectivity and morphology of the EC

The EC can also be divided into six layers (I-VI) as proposed originally by Ramon y Cajal (1901-02). Although many neuroanatomists maintain that there are only 5 layers as layer IV (otherwise known as lamina dissecans) is mostly acellular and is sometimes referred to as a plexiform layer (Insausti *et al.,* 1997). It separates layers III and V and is used as the demarcation between deep and superficial layers. In some species it can only be found in the more caudal regions of the EC, and therefore it may be considered a cell free subdivision of layer V. It has been suggested that the EC may be a transitional cortex, phylogenetically younger than the three layered hippocampus but older than the clearly 6 layered neocortex (Insausti *et al.,* 1997). Although termed the lamina dissecans, layer IV may contain large pyramidal cells with apical dendrites which send collaterals to the white matter and small cells which project locally (Witter *et al.,* 1989b). Layer I, which contains mostly fibres and few neuronal cell bodies, is also sometimes referred to as a plexiform layer (Insausti *et al.,* 1997), and represents the molecular layer of the EC. The neurones in this layer comprise spinous multipolar cells and horizontal cells (Germroth *et al.,* 1989) that ramify locally.

The remaining four are cellular layers. Layer II contains mostly large stellate neurones, with a smaller population of pyramidal cells. These are often grouped together in ‘cell islands’ although the layer becomes more continuous further caudally. This is less obvious in the rat than other species (Insausti *et al*., 1997). The spiny stellate cells of layer II, with many diverse and far-reaching projections, form the

main output from the EC to the hippocampus. A single layer II cell has been shown to project axonal branches towards the entire transverse DG (suprapyramidal and infrapyramidal blades, and the DG crest), the CA2-3 fields of the hippocampus, and the subiculum (Tamamaki and Nojyo, 1993). Their dendritic trees branch extensively throughout layers I and II and the superficial part of layer III, and many of the afferents in these layers will synapse with these cells. There is also limited evidence in some species to suggest that layer II stellate cells also project to layers V and VI of the EC (Ino *et al.,* 2000; Buckmaster *et al.,* 2004). Layer II also contains horizontal cells, differentiated from stellate cells by their horizontally orientated somata, dendrites and axons, which are restricted to the layer (Jones and Buhl, 1993; Germroth *et al.,* 1989). Finally, small pyramidal like cells can be found in layer II that ramify in the superficial layers.

Layer III is a broad layer containing large pyramidal neurones. These neurones have been broadly separated into two types depending on whether they project towards the angular bundle, ultimately to CA1 and the subiculum, or remain within the EC (Gloveli *et al.,* 1997). Within the EC, layer III neurones seem to collaterilise most abundantly within layer III, but may also project into both the deeper (V and VI) and the more superficial layers (I and II; Gloveli *et al.,* 1997). Collectively, layers I, II and III are often referred to as the superficial layers of the EC.

Layer V is a fairly dense cell layer containing mostly pyramidal neurones, with some horizontal and polymorphic neurones (Hamam *et al.,* 2000), and is thought to be the main origin of projections to the cortex (Dugladze *et al.,* 2001; Insausti *et al.,* 1997; Witter *et al.,* 1989a, b; Sorensen , 1985). Layer VI contains mainly pyramidal, bipolar or multipolar neurones (Gloveli *et al.,* 2001) and is less dense than layer V. This layer is also less distinct and often merges with both layer V and the white matter (Insausti *et al.,* 1997). In both layer V and VI by far the most abundant cell type are the pyramidal neurones. They are the principal neurones which project to various cortical and subcortical structures as discussed in the next section. These cells may also project to the DG, to relay information to the hippocampus, or to the superficial layers to influence network activity of the EC (Gloveli *et al.,* 2001). Other cells within layers V and VI also send collaterals to the superficial layers and the dentate granule cells or remain localised to the deeper layers. A summary diagram of the morphology and basic intrinsic connectivity can be seen in Figure 1.2.

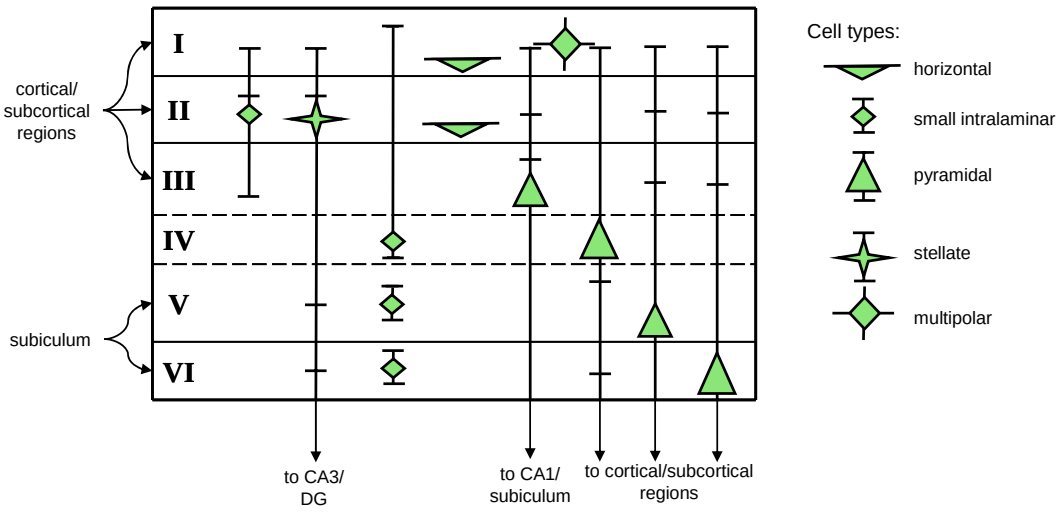


Figure 1.2. Schematic representation of structure and connectivity of the medial EC *including information on cell type and morphology in each layer.*

1.2.3. Extrinsic connectivity

The EC is closely linked to the hippocampus and is considered to act as a gateway between the hippocampus and the neocortex. The main input to the hippocampus from the EC is known as the perforant pathway and predominantly arises from stellate neurones in layer II of the EC. Although these also synapse with neurones of Ammon's horn (one of the two interlocking gyri composing the hippocampus) and the subiculum (Lopes da Silva *et al.,* 1990), the axons of the perforant pathway primarily target the molecular layer of the DG, where 85% of the total synapses are from the EC (Matthews *et al.,* 1976). Axons from the medial EC in rats primarily innervate neurones located in the middle third of the molecular layer of the DG. Axons from the lateral EC terminate in the outer third of the DG (Wyss, 1981). Additionally, the EC also projects to the contralateral hippocampus via the hippocampal commisure (Amaral *et al.,* 1984).

The perforant pathway is the first step of the trisynaptic loop. This refers to the polysynaptic circuit consisting of: 1. Axons primarily from layer II EC neurones that make excitatory synapses onto granule cells in the outer molecular layer of the DG (although there is some evidence for input to the DG from layer V neurones; Deller *et al.,* 1996; Witter *et al.,* 1988; Köhler, 1985). 2. Axons from the dentate granule cells, which form the mossy fibre pathway that synapse onto CA3 pyramidal cells. 3. The Schaffer-collateral commissural pathway, which consists of axons from CA3

pyramidal neurones (and contralateral CA1 pyramidal neurones, via the fornix) synapsing on CA1 pyramidal cells (Witter *et al.,* 1993). All projections in the perforant pathway are glutamatergic and excitatory (Andersen 1975; Misgeld, 1988), although in each region there are local GABAergic interneurones that provide feedforward and feedback inhibition (Freund and Buzsáki, 1996; Lacaille *et al*., 1987; Ribak and Seress, 1983; Woodson *et al.,* 1989). Most of the outputs from the hippocampus return to the EC from CA1 via the subiculum, where they terminate mainly in the deeper layers of the medial EC (Lopes da Silva, 1990). In the rat the output to the EC is strictly ipsilateral (Köhler, 1986), whereas there is a contralateral component in monkeys (Amaral *et al.,* 1987).

Not all inputs to the hippocampus from the EC are via the DG. There is also a monosynaptic pathway that terminates directly in the CA1 region, bypassing the first two stages of the trisynaptic loop. This is known as the temporoammonic (TA) pathway (Maccaferri and McBain, 1995), or the direct perforant pathway/entorhinal projection. The TA pathway primarily originates from the pyramidal cells of layer III of the EC (Steward and Scoville, 1976), and terminates on CA1 neurones. Greater than 90% of the axons terminate on pyramidal cells. However, they have also been shown to synapse onto inhibitory basket and chandelier neurones (Kiss *et al.,* 1996) and interneurones of the stratum lacunosum-moleculare (Lacaille and Schwartkroin, 1988). The direct projection from layer III neurones is also accompanied by a direct projection from layer II neurones to CA3 (Hjorth-Simonsen and Jeune 1972; Steward, 1976; Steward and Scoville, 1976). There is some debate as to whether the TA pathway is primarily excitatory or inhibitory (Soltesz and Jones, 1995). Initially it was thought the primary function of the direct TA pathway was to modulate information processed via the trisynaptic pathway (Yeckel and Berger, 1990). However, evidence has accumulated to suggest that the TA pathway actually transmits the information required for some hippocampal-dependent functions (Jarrard *et al.,* 1984; McNaughton *et al.,* 1989; Brun *et al.,* 2002).

The EC is also reciprocally connected to various association cortices (prefrontal, limbic and parieto-occipital-temporal) either directly or via the perirhinal cortex (Witter and Groenewegen, 1984). The EC sends and receives direct and strong inputs from the olfactory bulbs (Van Groen *et al.,* 1987). Other cortical regions with which the EC has strong connections include the prelimbic, infralimbic, dorsal peduncular

and the orbitofrontal and olfactory cortices. Weaker connections have also been found between the EC and the insular, cingulate, retrosplenial and area Fr2 of the medial frontal cortices (Insausti *et al.,* 1997; Condé *et al.,* 1995). In addition, sparse connections exist between the EC and somatosensory, auditory, visual and motor cortices (Insausti *et al.,* 1997). The majority of these outputs to the neocortex have been found to originate in layer V of the EC (Dugladze *et al.,* 2001; Insausti *et al.,* 1997; Witter *et al.,* 1989a, b; Sorensen, 1985). Afferents from neocortical layer V terminate mainly in layers I and II of the EC (Insausti *et al.,* 1997; Witter, 1993; Felleman and Van Essen, 1991). Subcortical regions, including the basal forebrain, thalamic nuclei, raphé nuclei, locus coeruleus, ventral tegmental area and amygdala also innervate various layers of the EC (Insausti *et al.,* 1997).

In summary, visual, auditory and somatosensory information, acquired and processed in polymodal association cortices, is conveyed via the parahippocampal and perirhinal cortices to the EC, DG, hippocampus and subiculum in a serial manner. Hippocampal output is then directed back to the EC, which sends reciprocal efferents to parahippocampal and perirhinal cortices to feed back information to the neocortical association areas. Thus, the EC is a relay station between the hippocampal and neocortical areas, acting as both an input and output processor. A summary diagram of the extrinsic connectivity of the EC is presented in Figure 1.3.

1.2.4. The EC and memory

The hippocampus has long been considered to be intimately involved in learning and memory and since the connectivity between the hippocampus and the EC is extensive it has also been suggested that the EC must also be involved. Together with other regions of the medial temporal lobe, the EC appears to be of particular importance for establishing long-term memory for facts and events (declarative memory; Squire and Zola-Morgan, 1991). The importance of the role of the EC in memory is supported by EC lesion studies, in which deficits in various learning and memory tasks have been identified (Roof *et al.,* 1993; Glasier *et al.,* 1995; Cho and Jaffard, 1995; Kopniczky *et al.,* 2006). In addition, studies in which entorhinal afferents from the presubiculum (Liu *et al.,* 2001) or from the perirhinal cortex (Liu and Bilkey, 1998) are selectively removed, suggest that the EC may serve a more critical function in the acquisition of declarative memory than was previously assumed.

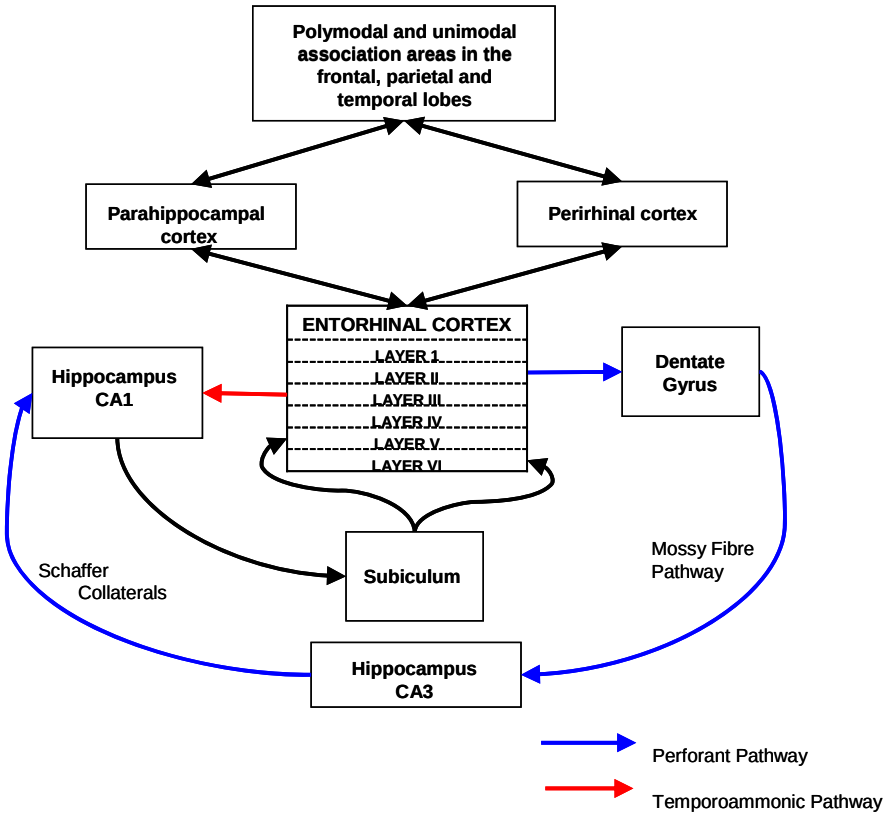


Figure 1.3. Simplified diagram showing the connectivity of the EC *with both the neocortex and the hippocampus (and related structures), highlighting the perforant and temporoammonic pathways.*

The role of the EC in memory is also frequently examined in humans and animals with medial temporal lobe damage, including Alzheimer's disease patients, whose early cognitive impairments can be traced to lesions in the EC (Van Hoesen *et al.,* 2000), and TLE patients. Recently, important evidence has come to light that has helped to elucidate how the EC contributes to hippocampal function and memory. Fell *et al.* (2001) showed that successful memory formation is accompanied by an enhancement in gamma frequency synchronisation between the rhinal cortex and the hippocampus. This led them to suggest that formation of declarative memories requires a direct entorhinal-hippocampal interaction. Frank and Brown (2003) investigated sustained activity in layer V of the EC, which they believe may be involved in maintaining representations to allow time for synaptic plasticity to occur, thus enabling the hippocampal-cortical circuit to form memories. Hafting *et al.* (2005) showed that the dorsal medial EC contains a directionally orientated topographically organised neural map of the spatial environment made up of what they referred to as "grid cells". They suggested that these grid cells may be responsible for aspects of spatial learning that had previously been attributed to the hippocampus.

1.3. The EC and CNS disorders

The EC has been implicated in a number of pathological conditions. Disruption of EC function is thought to be involved in the cognitive decline in various dementias including Alzheimer's disease, Parkinson's disease and Pick's disease (Braak and Braak, 1992; Braak *et al.,* 2000). Most strikingly, the morphological changes associated with Alzheimer's disease occur in the EC in the earliest stages of the disease, before symptoms have even begun to occur (Braak and Braak, 1992). In addition, it has been suggested that neuropathological changes in the EC may underlie a vulnerability to schizophrenia (Kurachi, 2003). However, the disease most commonly associated with dysfunction of the EC is TLE, which is the cause of seizures in 40% of adult epilepsies (McNamara, 1992; Schwartz *et al.,* 2000; Jones and Woodhall, 2005). The evidence for linking EC dysfunction with both schizophrenia and TLE is discussed in further detail below. It is interesting to note that there is a close association between schizophrenia and TLE. There is a higher prevalence of schizophrenia-like symptoms in epilepsy patients compared to the general population (Bredkjaer *et al.,* 1998; Sachdev, 1998; Schwartz and Marsh, 2000; Gaitatzis *et al.*, 2004), and people with a history of epilepsy have nearly 2.5 times the risk of developing schizophrenia (Qin *et al.,* 2005). Thus, schizophrenia and TLE may well share dysfunction of the same neuronal networks.

1.3.1 The EC and schizophrenia

Schizophrenia is a chronic, debilitating psychiatric illness affecting approximately 1% of the general population. It is characterized by two kinds of symptoms; positive psychotic symptoms - thought disorder, hallucinations, delusions, and paranoia - and negative symptoms – impairment in emotional range, energy, and enjoyment of activities. It has been suggested that disruption of the interactions between prefrontal and entorhinal/hippocampal areas may underlie many of the symptoms of the disease (Grace, 2000). Structural magnetic resonance imaging has identified changes in EC volume associated with schizophrenia, with volume positively correlating with severity of delusions (Prasad *et al.,* 2004). In addition, diffusional anisotropy (measuring myelination) suggests large-scale disruption of entorhinal connectivity (Kalus *et al.,* 2005). Displacement or absence of stellate cell clusters in layer II is common in post-mortem tissue (Beckmann and Senitz, 2002; Falkai *et al.,* 2000;

Kovalenko *et al.,* 2003). At the cellular level, changes in mRNA for synaptic markers such as GluR3, NR1 and synaptophysin, have been reported in post-mortem EC from schizophrenic patients (Arnold, 2000; Hemby *et al.,* 2002). Cholecystokinin (CCK) mRNA levels are reduced in layers III and V, where a decrease in inhibitory interneurones was also seen (Bachus *et al.,* 1997).

Significant learning and memory deficits are also seen in patients with schizophrenia (Aleman *et al.,* 1999; Rushe *et al.,* 1999; Paulsen *et al.,* 1995). Changes in dentate granule cell morphology and genetic markers indicative of changes in input from the EC have been reported (Lauer *et al.,* 2003; Austin *et al.,* 2004), and it has been suggested that these changes may underlie episodic memory deficits (Talamini *et al.,* 2005). In addition, sensorimotor gating deficits are common in schizophrenia (Geyer and Braff, 1987) and the severity of deficits correlates with the severity of psychosis (Meincke *et al.,* 2004). Importantly, the EC has been implicated as a key area involved in sensorimotor performance (Swerdlow *et al.,* 2001; Davachi and Goldman-Rakic, 2001).

The cognitive and affective symptoms of schizophrenia have been suggested to arise from an inability to integrate or synchronise activity in neuronal networks. The disorder is increasingly being viewed as arising from abnormal neuronal synchrony in cortical networks. Specifically, a number of studies have shown disruption of gamma rhythmogenesis in cortical networks in schizophrenic patients (e.g. Spencer *et al.,* 2003; Symond *et al.,* 2005). Harrison *et al* (2003) developed Lysophosphatidic acid 1 receptor (LPA1R) knock-out mice as a possible model for schizophrenia. LPA1R is a G-protein coupled receptor for the bioactive phospholipid, lysophosphatidic acid. The role of LPA1R is unclear, although it is thought to be involved in neurogenesis or neuroblast migration during development due to its embryonic expression profile (Harrison *et al*., 2003). LPA1R knockout mice show a deficit in prepulse inhibition, widespread changes in the levels and turnover of the neurotransmitter 5-HT, region-specific changes in levels of amino acids, and craniofacial dysmorphisms (Harrison *et al.*, 2003; Roberts *et al.*, 2005), changes which have been associated with schizophrenic-like pathology. Interestingly, in this model there is a selective loss of gamma frequency oscillations in superficial layers of the EC accompanied by a reduction in inhibitory control of principal cells (Cunningham *et al.,* 2004). These

studies lend support to the idea that disruption of EC function may be an underlying factor in schizophrenia.

1.3.2. The EC and epilepsy

Epilepsy, characterised by recurrent spontaneous seizures, is the most common neuropathological disorder in the world. Of the 450 million people with mental and neurological disorders worldwide, an estimated 50 million have epilepsy (Shorvon, 1990). There are over 40 different types of epilepsy, divided into symptomatic, idiopathic or cryptogenic categories and further subdivided into generalised or partial seizures (McCormick and Contreras, 2001). TLE is the most common form of partial epilepsy in adults (McNamara, 1992). Although the major focus has been on the hippocampus as the main site of TLE (Bradford, 1995; McCormick and Contreras, 2001), there is increasing evidence that the EC is heavily involved. Seizures can arise independently or preferentially in the EC (Rutecki *et al.,* 1989; Spencer and Spencer, 1994; Alarcon *et al*., 1997; Assaf and Ebersole, 1997; Assaf *et al*., 2003; Bartolomei *et al*., 2004, 2005). Focal seizures arising in the EC or the amygdala are more likely to result in clinical manifestations, whereas seizures originating in the hippocampus do not (Wennberg *et al.,* 2002). It has long been accepted that epilepsy involves abnormal and excess synchronous activity within neuronal networks. In this case, extreme hypersynchrony results in a pathologically oscillating network, to the extent where it is incapable of meaningful information processing. Fast cortical/gamma oscillations (30-100 Hz) are commonly recorded in states of active alertness. They are thought to be important in sensory processing. Specifically evidence suggests that neurones which represent the same object or event fire in temporal synchrony which groups features (referred to as ‘sensory/feature binding’) allowing the formation of coherent object representations (for review see Tallon-Baudry and Bertrand, 1999). Intensified gamma activity has been detected in the EEG of epileptic patients (Hirai *et al.,* 1999; Willoughby *et al.,* 2003). This increased and extremely synchronous gamma activity, is though to represent a pathological state of excessive (beyond normal physiological limits) temporal binding in the neural networks or ‘over-binding’ (Medvedev**,** 2001) and is suggested to be a “prerequisite to the development of seizures” (Willoughby *et al.,* 2003). *In vivo* studies in rats with chronically induced epilepsy have demonstrated increased high frequency events and oscillations in the EC, with a high degree of similarity to events recorded in human TLE patients (Bragin *et al*., 2002, 2004).

In human epilepsy as many as 30% of patients are refractory to available drug treatments (Sander, 1993; Kwan and Brodie, 2000). Control of drug-resistant TLE by surgical resection involves a partial temporal lobectomy, which invariably removes the EC (Sperling *et al.,* 1996). The success rate of this surgery appears to correlate with the amount of parahippocampus and EC removed during the resection (Siegel *et al.,* 1990; Fried, 1993; Sperling *et al.,* 1996). Goldring *et al.* (1992; 1993) have suggested that the removal of the EC is vital for surgical control of refractory TLE. One of the symptoms of TLE is temporal lobe atrophy, as seen in MRI studies with epileptic patients. Many MRI studies have shown that the EC is significantly reduced in volume in TLE patients, often without an accompanying loss of hippocampal volume (Bernasconi *et al.,* 1999; 2001; Jutila *et al.,* 2001; Bonilha *et al.,* 2003). Lesions of the EC are mainly localised to layer III (Schwarcz and Witter, 2002; Du *et al.,* 1993). EC atrophy is specifically associated with TLE, none is evident in epilepsies arising outside of the temporal lobe, or in generalised epilepsies of unknown aetiologies (Bernasconi *et al.,* 2003). Research using surgically resected tissue has also shown atrophy (Du *et al.,* 1993) and gliosis (Yilmazer-Hanke *et al.,* 2000) in the EC. In addition, a recent study has identified changes in several genes in the EC of patients which indicated that local dysregulation of neurotransmission in the EC is common in human TLE (Jamali *et al.,* 2006).

Epileptic activity in rats is characterized by electrographic seizure activity that may arise in the EC before the hippocampus (Ben-Ari *et al.,* 1981; Collins *et al.,* 1983; Stringer, 1994). Studies in various models have shown that long lasting ictal-like epileptiform discharges generated in the EC entrain hippocampal neurones via the trisynaptic loop (Du *et al.,* 1995; Jones and Lambert, 1990a; Avoli *et al.,* 2002). Such discharges are thought to originate in deep layers, particularly layer V (Jones and Lambert, 1990a, b; Avoli *et al.,* 1996; Lopantsev and Avoli, 1998) as the deeper layers of the EC are more susceptible to seizures, whereas the more superficial layers appear to be seizure resistant (Jones, 1993). However, a recent study by Tolner *et al.* (2005) contradicts this idea. Their results suggest that epileptiform activity is more readily generated in the superficial layers of the medial EC, although they agree that this would lead to an increased excitatory drive onto the hippocampus. There is some evidence to support control by CA3 over the generation of spontaneous epileptiform activity in the EC (Avoli, 2001; Stoop and Pralong, 2000; Barbarosie and Avoli,

1997). The absence of control of ictal discharge generation in EC by CA3 may contribute to the higher seizure susceptibility in young animals (Calcagnotto *et al.,* 2000; Cavalheiro *et al.,* 1987; Hablitz, 1987). Significant neuronal loss in the EC has also been seen in animal models (Wozny *et al.,* 2005; Du *et al.,* 1995), also mainly localised to layer III. The EC atrophy in animal models induced by injection of amino-oxyacetic acid (AOAA) seems to cause neurones in the superficial layers of the EC to become hyperexcitable, perhaps due to synaptic reorganisation (Scharfman *et al.,* 1998). Recently, using laser scanning photostimulation it was shown that there is reduced recurrent inhibition of layer II stellate neurones in epileptic animals (Kumar *et al.,* 2007). The hyper-excitability in layer II may be due to a loss of GABAergic interneurones in layer III (Kumar and Buckmaster, 2006). There is also thought to be decreased inhibition in layer V in pilopcarpine treated rats (de Guzman *et al.,* 2008), which may contribute to network epileptic hyper excitability in this model of TLE.

1.4. Physiology and pathophysiology of layer III

Information on the normal physiology and pharmacology of cells in layer III is limited, particularly when compared to the wealth of information on layer II and, to some extent, layer V (Jones and Heinemann, 1988; Jones, 1993; Berretta and Jones, 1996a; b; Heinemann *et al.,* 2000; Jones and Woodhall, 2005; Evans *et al.,* 2000; 2001; Bailey *et al.,* 2004; Woodhall *et al.,* 2005). Gloveli *et al.* (1997) identified four cell types in layer III of the medial EC, using biocytin to study their morphology, and intracellular recordings to determine the basic electrophysiology. Neurones in layer III were largely pyramidal cells, differing in their dendritic arborisations and whether they projected outside of the EC, or were 'local circuit' neurones. Using various pharmacological agents they provided evidence for NMDAr and non-NMDAr-mediated excitation and $GABA_A$r and $GABA_B$r-mediated inhibition present in layer III neurones, although these were not present in all cell types. They put specific emphasis on the excitability of the network in the presence of bicuculline, suggesting that this indicated a strong $GABA_A$r-mediated inhibition in this layer of the EC. Neurones in layer III of the lateral EC have been found to be similar to those in the medial EC (Tahvildari and Alonso, 2005). Van der Linden and Lopes da Silva (1998) also carried out intracellular current-clamp recordings in the medial EC to compare layer III neurones to those in layer II. They described two cell types according to the presence of a time-dependent inward rectification. This manifests as a depolarising

sag in the membrane back towards resting membrane potential in response to hyperpolarisation, which is caused by the activation of a mixed cation current called I_h or "SAG". Neurones in layer III, in direct contrast to those in layer II, were found to be mostly of the "NO SAG" type, which may indicate that neurones in layer III are less excitable than layer II neurones (Van der Linden and Lopes da Silva, 1998). Dickson *et al.* (1997) found neurones in layer III to typically be regularly spiking cells, which, unlike layer II neurones, lacked subthreshold oscillations. However, they also found that layer III neurones showed pronounced excitability that they felt may be related to the degeneration that is seen in layer III in epilepsy (described in more detail below).

Dhillon and Jones (2001) investigated recurrent EPSPs in layer III neurones. These were found to contain a fast component mediated by AMPAr/KAr and a slow component mediated by NMDAr. The recurrent EPSPs in layer III neurones were larger in amplitude than those found in layer V, whilst no recurrent EPSPs were seen in layer II (Dhillon and Jones, 2001). A number of presynaptic receptors have been found in layer III including dopamine receptors (Stenkamp *et al.,* 1998) and serotonin receptors (5HT1A; Schmitz *et al.,* 1998) on glutamatergic terminals and $GABA_Br$ on GABAergic terminals (Gloveli *et al.,* 2003). Finally, various frequencies of oscillatory activity have been induced in layer III neurones including gamma frequency induced by KA (Cunningham *et al.,* 2003), theta frequency induced by physostigmine (Alonso and Garcia Austt, 1987) and SWO, at a frequency of < 1Hz, which can be induced in adult animals by reducing extracellular magnesium (Cunningham *et al.,* 2006b).

The relative lack of knowledge on the physiological properties of neurones in layer III is surprising in the light of increasing evidence for a role in TLE. In particular, it has been shown that there is a characteristic pattern of neuronal loss and gliosis in layer III in a number of animal models of the disease (Du and Schwarcz, 1992; Du *et al.,* 1995; Tolner *et al.,* 2005), where it has been shown that greater than 50% of the total neuronal population may be lost. A similar situation may occur in human patients with TLE (Du *et al.,* 1993), although, a recent study (Dawodu and Thom, 2005) has suggested that selective laminar neuronal loss in the EC is less common in patients than animal models. Du and Schwarcz (1992) proposed that the loss of layer III pyramidal cells led to the destruction of the TA pathway to the CA1 region, whilst the perforant path from layer II to the DG was relatively preserved. As the TA pathway is

believed to primarily innervate interneurones, this would lead to decreased inhibition in CA1 and production of a 'seizure prone' circuit, as this would directly reactivate the EC (Denslow *et al.,* 2001). A recent study of entorhinal-hippocampal pathways in pilocarpine-treated rats (Ang *et al.,* 2006) also reported that the TA pathway was severely disrupted, with concurrent sparing of the perforant pathway to the DG. Although the TA pathway changed from primarily inhibitory to powerfully excitatory, the authors proposed that pyramidal cell loss from layer III (or indeed CA1) would actually dampen this effect. In agreement, Kumar and Buckmaster (2006) have provided evidence that the cells lost from layer III are GABAergic interneurones not pyramidal cells, and that loss of these neurones would decrease feed-forward inhibition of layer II, causing hyperexcitability in layer II stellate cells and hence the perforant path.

1.5. Kainate receptors

There are two main families of glutamate receptor, mGlur, which are made up of seven transmembrane domains and mediate their responses via G-proteins that initiate a chain of events inside the cell and iGlur, which are ion channels that open in response to the transmitter. iGlur are principally permeable to sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) ions and belong to one of three families, NMDAr, AMPAr and KAr. There is a wealth of information available on the structure, location, properties and function of NMDA and AMPAr (for reviews see Wisden and Seeburg, 1993; Jonas, 1993; Borges and Dingledine, 1998; Dingledine *et al.,* 1999; Yamakura and Simoji, 1999; Cull-Candy *et al*., 2001, 2006; Gouaux, 2004; Mayer, 2005; Kohr, 2006; Malinow and Malenka, 2002). Much less is known about the function and physiological roles of KAr in the CNS, and this has largely been due to the lack of pharmacological agents able to discriminate between AMPAr and KAr.

KA was first isolated from seaweed more than 50 years ago. Davies and Watkins (1981) were the first to propose that KA activated a distinct class of iGlur, in agreement with the evidence of KA binding sites present in the rat CNS (London and Coyle, 1979). However, this was confused by the discovery that AMPAr could also be activated by KA (Hollmann *et al.,* 1989). It was not until the early 1990s, after cloning of the KAr subunits, that they were unequivocally shown to exist (Bettler *et al.,* 1990;

1992; Egebjerg *et al.,* 1991, Herb *et al.,* 1992; Sakamura *et al.,* 1992; Werner *et al.,* 1991).

1.5.1. Subunit composition and expression

KAr are tetramers, consisting of four subunits, from a possible five (Hollmann and Heinemann, 1994), termed GluR5, GluR6, GluR7, KA1 and KA2. GluR5-7 share 70% sequence homology with each other (Bettler *et al.,* 1990; 1992; Egebjerg *et al.,* 1991; Sommer *et al.,* 1992), as do KA1 and KA2. However, the KAr subunits show only 40% similarity to the GluR subunits (Werner *et al.,* 1991; Herb *et al.,* 1992; Sakimura *et al.,* 1992). All five subunits also share some sequence homology with both AMPAr (~30%) and NMDAr (~10%) subunits. Like all iGlur subunits, the KAr subunits fold to form three trans-membrane domains and one p-loop, which enters the plasma membrane from the cytoplasm to form the receptor pore (Bennett and Dingledine, 1995; See Figure 1.4.).

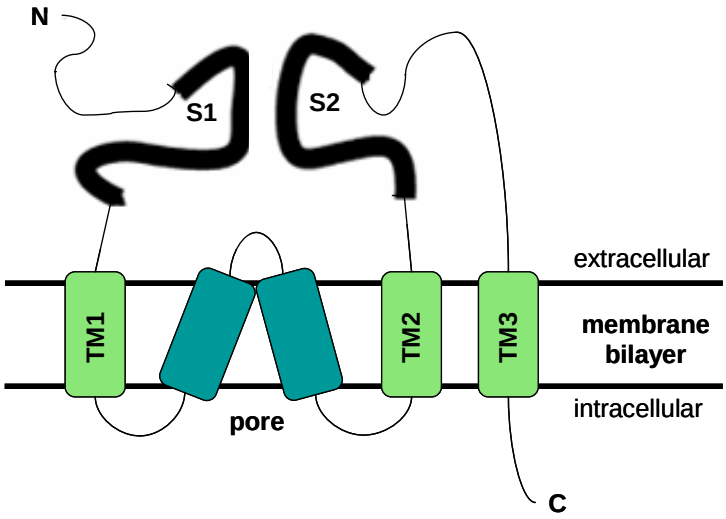


Figure 1.4. Schematic representation of iGluR subunit topology*. (The S1 and S2 domains form the ligand binding domain)*

Expression studies in both *Xenopus* oocytes and HEK 293 cells have shown that the GluR subunits are capable of forming homomeric (Bettler *et al.,* 1991; Egebjerg *et al.,* 1991; Sommer *et al.,* 1992; Schiffer *et al.,* 1997) and heteromeric (Bortolotto *et al.,* 1999; Cui and Mayer, 1999; Paternain *et al.,* 2000), low affinity, ligand-gated ion channels. KA1 and 2 have been shown to form high-affinity homomeric binding sites (Werner *et al.,* 1991; Herb *et al.,* 1992), but they require partnership with at least one GluR subunit in order to become a functional receptor (Herb *et al.,* 1992; Sakimura *et*

al., 1992). Both low and high-affinity binding sites have been found in the CNS of various species, including rat and human (London and Coyle, 1979). Contractor *et al.* (2003) proposed that higher-affinity binding sites contain a KA subunit whereas the lower affinity sites do not. However, expression studies have shown that GluR5/KA2 heteromers actually have a lower binding affinity than either GluR5 or KA2 homomers (Herb *et al.,* 1992).

Various groups have mapped the distribution of KAr mRNA expressing cells in the rat CNS (Wisden and Seeburg, 1993; Bahn *et al.,* 1994; Tölle *et al.,* 1993) and have shown that GluR5-7 and KA2 are prominently expressed throughout the CNS including in the cortex, the striatum, the cerebellum and the hippocampus, but that KA1 is not so widely expressed. In the rat neocortex all KAr subunits, with the notable exception of KA1, are expressed by E14 (Bahn *et al.,* 1994). Although this generally remains true through to the adult, there are significant changes in expression levels through development. Specifically, expression of the GluR5 subunit tends to decrease after P12, although this subunit also has the largest variation, at any given age, between different regions of the cortex (Bahn *et al.,* 1994).

There is little information available concerning specific expression patterns of KAr or their subunits in the EC. Bahn *et al* (1994) indicated that KAr subunit expression in the EC is broadly the same as the rest of the cortex, with a couple of notable exceptions. Expression of KAr in the EC appears later than the rest of the cortex, with no expression seen until E19 (Bahn *et al.,* 1994). Also, GluR5 subunit expression is never as high as is seen in other cortical regions (Bahn *et al.,* 1994). Like all other areas of the cortex, KA1 is hardly expressed in the EC throughout development and in the adult rat, whereas KA2 is one of the more highly expressed subunits (Bahn *et al.,* 1994; Wisden and Seeburg, 1993). Kohama and Urbanski (1997) used a pan-GluR5/6/7 antibody, and showed dense expression across the whole of the primate EC, with little indication of lamina selectivity. A recent *in vitro* hybridization study has suggested a considerable variation in expression of KAr subunits in the human EC (Beneyto *et al.,* 2007). GluR7 showed the densest level of expression, with intermediate levels of GluR6, KA1 and 2, but only low levels of GluR5. GluR7 also showed the most prominent lamina-selectivity with particularly high levels in layers V and II/III.

1.5.2. RNA editing and alternative splicing

KAr are mostly permeable to Na^+ and K^+ ions with a low conductivity for Ca^{2+} (Ferrer-Montiel and Montal, 1996). Ca^{2+} permeability is determined by mRNA editing. GluR5 and GluR6 are the only KAr subunits that have been shown to undergo mRNA editing. Editing of a glutamine (Q) residue in the pore of these subunits to an arginine (R) results in a change in permeation properties (Sommer *et al.,* 1991). Unedited versions of the subunits display inwardly rectifying currents as the outward currents are blocked by the presence of intracellular polyamines. However, mRNA editing causes the I-V relationship to become linear (Bowie and Mayer, 1995; Kamboj *et al.,* 1995; Bähring *et al.,* 1997). Editing can also occur at the Q/R site of the trans-membrane domain 2, which confers a change in single channel conductance and Ca^{2+} permeability i.e. unedited receptors exhibit higher Ca^{2+} permeability (Burnashev *et al.,* 1996) and a higher unitary conductance (Swanson *et al.,* 1996) when compared to receptors that include one or more edited subunits. The GluR6 subunit also has two additional mRNA editing sites located in the trans-membrane domain 1, which also effect calcium permeability (Köhler *et al.,* 1993), fully edited GluR6 subunits are left essentially impermeable to Ca^{2+}.

As well as mRNA editing, the GluR subunits can also be alternatively spliced to give further diversity (see Figure 1.5). All subunit diversity arising from alternative splicing of the KAr subunits has been found to play an important role in subunit trafficking (Ren *et al*., 2003b). The GluR5 subunit has been shown to be alternatively spliced in its long extracellular N terminus, giving the variants referred to as GluR5-1 and GluR5-2, which differ in the inclusion of a sequence of 15 amino acids. In addition, there is also alternative splicing of the GluR5 C-terminal domain, which gives rise to a further three main variants termed GluR5a, b and c (Sommer *et al.,* 1992) and a further variant, GluR5d, which is only present in humans (Barbon *et al.,* 2001). GluR6 is also alternatively spliced in the C-terminus giving two main splice variants, GluR6a and b (Gregor *et al.,* 1993). There is a further variant, GluR6c, which involves the insertion of 15 amino acids in the C terminal domain, and which is only found in humans (Barbon *et al.,* 2001). GluR7 also has two functional splice variants, GluR7a and b (Schiffer *et al.,* 1997). All variants of the GluR subunits have a conserved sequence of 16 amino acids in the C terminal domain, found close to the

last trans-membrane domain. As yet KA1 and KA2 have not been shown to undergo any splicing or RNA editing.

In addition, several groups have provided evidence for the phosphorylation of KAr subunits, which may regulate function (Wang *et al.,* 1993; Ghetti and Heinemann, 2000). GluR6 has been shown to interact with the PDZ domain of several modulatory proteins (Garcia *et al.,* 1998; Hirbec *et al.,* 2003), and this is probably involved in receptor trafficking and targeting, as discussed below.

1.5.3. KAr pharmacology

As already mentioned, pharmacological investigation of KAr has been somewhat hampered by a lack of selective tools. Until recently, there was difficulty in distinguishing between KAr and AMPAr mediated currents, as even KA was found to activate AMPAr (Patneau and Mayer, 1991) and, similarly, AMPA was found to activate KAr (Herb *et al.,* 1992). However, the development of AMPAr selective, non-competitive antagonists such as GYKI 53655 (Paternain *et al.,* 1995), has allowed better examination of KAr function, although these antagonists have not been tested against all KAr subunits. Studies have shown that the various native KAr have very different physiological and pharmacological properties (Wilding and Huettner, 2001). KAr are activated by KA with an EC_{50} of 6–23 µM (Heuttner, 1990) and can be blocked by CNQX, the archetypal non-NMDAr antagonist, once AMPAr have been blocked (Wilding and Huettner, 1995; Paternain *et al.,* 1996).

Synaptic responses that are mediated by KAr share two main features; the synaptic current is smaller than that mediated by AMPAr (about 10% of the total peak current) and it has significantly slower deactivation kinetics (Castillo *et al.,* 1997; Vignes *et al.,* 1997; Frerking *et al.,* 1998). The slow decay kinetics of these receptors has been shown to be an intrinsic property of the receptors (Bureau *et al.,* 2000; Kidd and Issac, 2001) and not due to an extrasynaptic location as previously suggested (Lerma, 1997). Activation of KAr is followed by either deactivation due to ligand unbinding or desensitization caused by closing of the ion channel pore while the ligand remains bound. KAr desensitize completely and rapidly in response to glutamate, with a time constant of approximately 5 ms (Dingledine *et al*., 1999). This profound desensitization, together with slow recovery, is thought to play an important role in

determining the frequency and amplitude of excitatory responses and may provide a mechanism to prevent excitotoxicity (Frandsen and Schousboe, 2003; Jones and Westbrook, 1996).

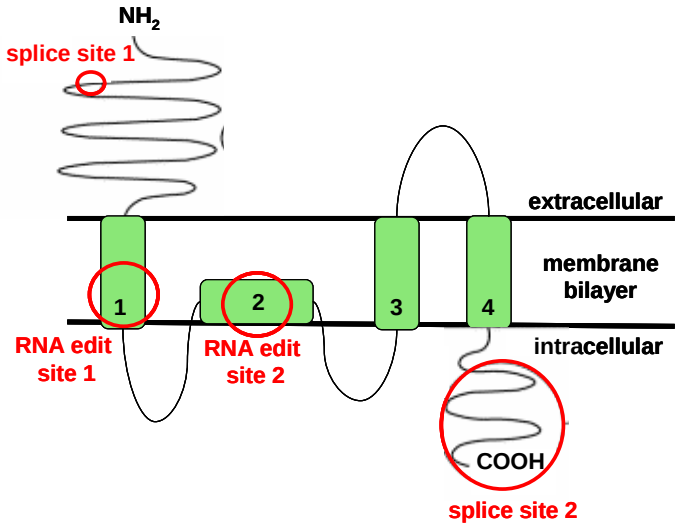


Figure 1.5. Alternative splicing and RNA editing of KAr subunits. *RNA edit site 1: RNA editing of GluR6 at I/V and Y/C sites in TM1. RNA edit site 2: RNA editing of GluR5 (residue 591) and GluR6 (residue 590) at the Q/R site in M2. Splice site 1: alternative splicing of GluR5 in the N-terminal domain. Splice site 2: alternative splicing of GluR5, GluR6 and GluR7 giving rise to C-terminal splice variants.*

As with AMPAr, agonist binding to KAr involves the N terminus and the extracellular loop between the last two trans-membrane domains (Armstrong *et al.,* 1998). However, the ligand binding cavity in GluR5 subunits is significantly larger than that of GluR2 (in AMPAr) or GluR6 subunits (Mayer, 2005). This has enabled the development of GluR5 selective ligands which are prevented from binding to other subunits by steric occlusion. These include the GluR5 selective agonist ATPA (which is also a partial agonist at GluR6/KA2 receptors; Clarke *et al.,* 1997) and UBP 302, which is the active *s* enantiomer of UBP 296, a potent, selective, competitive antagonist of all GluR5 containing KAr (More *et al.,* 2004). For a more comprehensive list of KAr ligands see Table 1.1.

Several allosteric modulators for KAr have been identified. Plant lectins (e.g. concanavalin A) are proposed to block desensitisation of KAr (Partin *et al.,* 1993). Also, some *cis* unsaturated fatty acids (e.g. arachidonic acid) block native KAr (Wilding *et al.,* 1998), specifically acting at edited GluR6 and GluR6/5 but only weakly inhibiting edited GluR5 or unedited subunits (Wilding *et al.,* 2005).

1.5.4. Trafficking and synaptic targeting

KAr are found both pre and postsynaptically in most regions of the CNS, and, as with the other iGlur, controlled, selective, subunit-specific synaptic targeting plays a critical role in modifying the strength of excitatory synapses. When compared to the trafficking of subunits of the NMDA and AMPAr, relatively little is known about KAr. However, recent studies have begun to shed light on distinct motifs that control the trafficking and expression of KAr at synaptic sites.

The most studied of the KAr subunits is the KA2 subunit. As mentioned previously, these are capable of forming non-functional homomeric complexes. Ren *et al.* (2003a) determined that these KA2 complexes remain in the endoplasmic reticulum unless they are co-assembled with a GluR subunit. ER retention is dependent on a motif of positively charged arginine residues in the C terminal region. However, the KA2 C terminus has also been found to contain an endocytic motif that causes rapid endocytosis of the receptor, maintaining a low level of expression of receptors containing KA2 at the plasma membrane. It has now been shown that alternative splicing of the C terminal region of both GluR5 and GluR6 strongly affects their plasma membrane expression (Ren *et al.,* 2003b; Jaskolski *et al.,* 2004). However, when trafficking motifs in the C terminus are disrupted, plasma membrane expression still occurs once the subunit has assembled with non-modified subunits, therefore, it would appear that oligomerisation may be the most crucial element of synaptic targeting (Yan *et al.,* 2004). Supporting this suggestion is evidence that GluR6a and GluR7a, which have high levels of plasma membrane expression due to forward trafficking motifs, can promote the expression of other subunits (Jaskolski *et al.,* 2004; 2005).

Compound	Activity	Selectivity	Reference
ATPA	agonist	GluR5, partial agonist at GluR6/KA2	Clarke *et al.,* 1997
(S)-5-Iodowillardine	agonist	GluR5, partial agonist at GluR6/KA2	Patneau *et al.,* 1992 Swanson *et al.,* 1998
LY 333494	agonist	GluR5	Small *et al.,* 1995
Dysiherbaine	agonist	GluR5 (but active at mGluR5)	Sakai *et al.,* 2001 Swanson *et al.,* 2002
LU 97175	Competitive antagonist	GluR7>GluR5>GluR6	Löscher *et al.,* 1999
LY 377770	Competitive antagonist	GluR5	O'Neil *et al.,* 1998 O'Neil *et al.,* 2000
LY 293884	Competitive antagonist	GluR5	O'Neil *et al.,* 1998
LY 382884	Competitive antagonist	GluR5	Simmons *et al.,* 1998
UBP 296	Competitive antagonist	GluR5, GluR5/GluR6, GluR5/KA2	More *et al.,* 2004
NS 3763	Non-competitive antagonist	GluR5	Christensen *et al.,* 2004
SYM 2081	antagonist	GluR5, GluR6 (Blocks EEAT2 at high concs.)	Zhou *et al.,* 1997
MSVIII – 19	antagonist	GluR5	Sanders *et al.,* 2005

Table 1.1. **KAr *pharmacology.*** *Adapted from Pinheiro and Mulle (2006).*

Additionally, several KAr binding proteins have been found that have been implicated in trafficking and synaptic targeting, including PDZ domain-containing proteins. SAP97 and PSD 95 cause clustering of KAr (Garcia *et al.,* 1998) and GRIP, PICK1 and Syntenin associate with GluR5b, GluR5c and GluR6a regulating function and synaptic stability of KAr (Hirbec *et al.,* 2003). GRIP and PICK1 are not specific to KAr (they are also involved with AMPAr), although they do appear to mediate distinct actions. For example, GRIP interactions increase AMPAr function whilst

decreasing KAr function (Hirbec *et al.,* 2002). It has also been shown that KAr interact with cadherin/catenin complexes, which may not be surprising as these are also known to interact with PDZ domain containing proteins, which have been found to play an important role in the initial recruitment and stabilisation of receptors at newly formed synapses (Coussen *et al.,* 2002). Coussen *et al.* (2005) have also shown that GluR6 interacts with other proteins involved in assembly and trafficking of the protein, including dynamin-1and NSF.

1.5.5. Postsynaptic KAr

Development of AMPAr selective antagonists such as GYKI 53655 has enabled the investigation into KAr to move forward, identifying them postsynaptically contributing to the synaptic current. Postsynaptic KAr were first shown to be present on CA3 pyramidal neurones (Castillo *et al.,* 1997), where high frequency stimulation of the mossy fibre pathway (in the presence of GYKI 53655 and the NMDAr antagonist, 2-AP5), unveiled a CNQX-sensitive current with slow rise and decay times. These KAr were believed to be heteromers containing a GluR5 subunit (as the response was blocked by LY 29355; Vignes *et al.,* 1997) and a GluR6 subunit (as the current was no longer present in GluR6 knockout mice; Mulle *et al.,* 1998). However, lack of high quality subunit-selective antibodies still hampers the confirmation of these functional studies with immunohistochemistry. It was postulated that these postsynaptic KAr were present extrasynaptically, requiring large amounts of glutamate to be released in order to spill out of the synapse, reflected by the requirement for repetitive stimulation (Mayer, 1997). However, PDC, a glutamate uptake inhibitor, has no effect on the kinetics of the KAr mediated current (Castillo *et al.,* 1997), suggesting that the slow kinetics are intrinsic properties of the receptors.

Since their discovery in the mossy fibre pathway, postsynaptic KAr have also been identified at a variety of other synapses, including in the somatosensory cortex (Kidd and Isaac, 2001) and the perirhinal cortex (Park *et al.,* 2006). They were also found on interneurones in the hippocampus, where there was no requirement to stimulate at high frequency, possibly indicating that the receptors are not extrasynaptic at these synapses (Cossart *et al.,* 1998). Also, more recently, a postsynaptic KAr-mediated current could be evoked using low frequency stimulation in the superficial layers of

the EC (West *et al.,* 2007). This study found that the current was significantly larger in the layer III cells than in layer II.

In almost all of the studies where postsynaptic KAr mediated currents have been identified (except in the squirrel retina – DeVries and Schwartz, 1999), the time constants for activation and desensitization of the receptors have been considerably different to what would have been predicted from results in cell culture. Although the reasons behind this discrepancy have yet to be elucidated, it has been suggested that this could be due to differing subunit composition of native KAr (Lerma *et al.,* 2001). In most cases it is assumed that postsynaptic KAr are present in the same synapses as other glutamate receptors, specifically AMPAr, although some synapses have been shown to solely contain KAr. For example, cortical cells were shown to express both AMPAr and KAr (Kidd and Isaac, 1999). However, this study also showed that although both KAr mediated slow responses and AMPAr fast responses were seen simultaneously, no slow KAr mediated tail was evident on the fast rising responses. This suggests that these receptors do not co-localise in single synapses. Kidd and Isaac (1999) also demonstrated that the ratio of synapse type changes during development, leaving less KAr synapses during the critical period for activity-dependent plasticity.

There is a role for KAr in synaptic plasticity at several synapses. In the somatosensory cortex it has been shown that when presynaptic stimulation of thalamo-cortical axons is paired with a depolarisation of the postsynaptic cell, KAr-mediated EPSCs undergo long term depression (Kidd and Isaac, 2001). Also, in the perirhinal cortex KAr can be removed from the postsynaptic membrane in an activity-dependent manner by a mechanism involving the interaction between the KAr and PICK1 (Park *et al.,* 2006). Finally, it has also been shown that NMDAr-independent synaptic plasticity in the mossy fibre pathway can be completely blocked following addition of a GluR5 subunit selective antagonist (Bortolotto *et al.,* 2004). It has been suggested that, due to their long decay times, a decrease in the KAr mediated EPSC would decrease the ability of the neurone to temporally summate inputs.

1.5.6. Presynaptic KAr

KAr are present on presynaptic terminals where they have been shown to be involved in the regulation of transmitter release. Both facilitation and inhibition of release, at

both excitatory and inhibitory synapses have been reported (Contractor *et al.,* 2000; Lauri *et al*., 2001; Min *et al*., 1999; Rodriguez-Moreno *et al.,* 1997).

In CA3 neurones in the hippocampus, frequency-dependent facilitation of NMDAr-mediated EPSCs (recorded in the presence of GYKI 53655) is inhibited in the presence of CNQX (Schmitz *et al.,* 2001) or LY 382884, the GluR5 subunit selective antagonist (Lauri *et al.,* 2001), without change in the response to a single stimulus. These results indicate that there is a presynaptic KA autoreceptor acting to facilitate the release of glutamate at these synapses. The presence of a presynaptic KAr was supported by initial transgenic knockout studies (Contractor *et al.,* 2001; Contractor *et al.,* 2003). However, GluR5 knockouts had no effect on paired pulse or frequency facilitation, implicating the GluR6 subunit, not GluR5, in direct contrast to the results using LY 382884. Subsequently, Pinheiro *et al.* (2005) have also implicated the GluR7 subunit, as GluR7 knockouts show severely disrupted paired pulse facilitation (PPF). Therefore, the most likely subunit composition of these autoreceptors is a GluR6/GluR7 heteromer.

The mechanism underlying the facilitation by the presynaptic KA autoreceptor is likely to be depolarisation of the terminal or direct Ca^{2+} influx through the receptors rather than any metabotropic effect due to the speed of the effect in high frequency (100 Hz) trains (Schmitz *et al.,* 2001). Ca^{2+} imaging supports depolarisation of the presynaptic terminals and activation of voltage-gated calcium channels (VGCC; Kamiya *et al.,* 2002). However, as the action of presynaptic KAr can also be impaired by philanthotoxin (Lauri *et al.,* 2003; Pinheiro *et al.,* 2007), which selectively targets unedited Ca^{2+} permeable glutamate receptors, it is likely that there is some contribution of direct Ca^{2+} influx. Presynaptic KA autoreceptors acting to facilitate transmitter release have also been found at synapses of cerebellar stellate and Purkinje neurones. Interestingly, in the stellate cells, but not Purkinje, this becomes a depression in glutamate release at higher frequency stimulation. This may be due to a difference in subunit composition (Delaney and Jahr, 2002). There is also evidence for the presence of presynaptic KAr facilitating glutamate release in the olfactory bulb (Davila *et al.,* 2007) and the neocortex (Campbell *et al.,* 2007).

Presynaptic KA autoreceptors also appear to inhibit glutamate release at some central synapses. For example, during early development, thalamocortical synapses in barrel

cortex show a depression of synaptic transmission at high frequency stimulation that is removed in the presence of the GluR5 selective antagonist, LY 382884 (Kidd *et al.,* 2002). Tonically active presynaptic KAr acting to depress glutamate release are also present at CA1-CA3 synapses in the hippocampus during early postnatal development, as LY 382884 increases the frequency of NMDA mediated mEPSCs in the presence of GYKI 53655 (blocking AMPAr). Unlike the KA autoreceptors that depress glutamate release in the barrel cortex, autoreceptors in the developing hippocampus appear to mediate their effects via a metabotropic action, as the effects of LY 382884 (and ATPA, the GluR5 agonist) were blocked by the addition of pertussis toxin (Lauri *et al.,* 2006). This metabotropic action mediated by ionotropic KAr was first shown in the regulation of GABA release (Rodriguez-Moreno and Lerma, 1998), as discussed below.

The first evidence for presynaptic KAr modulating GABA release was presented by Rodriguez-Moreno *et al.* (1997), who showed that in both cultured neurones and hippocampal slices, application of KA reduced the frequency and amplitude of mIPSCs. Rodriguez-Moreno and Lerma (1998) were the first to suggest a metabotropic action of ionotropic KAr because the ability of the receptor to inhibit GABA release in the hippocampus were dependent on the activation of a pertussis toxin (PTX)-sensitive G-protein, and suppressed by inhibitors of phospholipase C (PLC) and protein kinase C (PKC). Since then a wealth of evidence for a metabotropic action of presynaptic KAr, at both GABAergic and glutamatergic synapses, has been produced (reviewed in Rodriguez-Moreno and Sihra, 2007; see figure 1.6.).

The question of how KAr can interact with G-proteins to mediate metabotropic actions has been the source of much debate, as the topology of KAr is unlike that of the 7-transmembrane domain G-protein coupled receptors. However, Rozas *et al.* (2003) showed that the GluR5 subunit, is also able to activate a G protein that depresses VGCC and, therefore, may be responsible for the ability of KA to depress transmitter release. In addition, Ruiz *et al.* (2005) observed that KAr in CA3 neurones associate with G-proteins, as they are precipitated by an anti-GluR6 antibody that co-precipitates both GluR6 and KA2 subunits. Regardless of this, the mechanisms and functional significance of a KAr with both ionotropic and metabotropic modes of action remain elusive, although it is interesting to note that AMPAr have also been shown to mediate effects via G-proteins (Wang and Durkin, 1995; Wang *et al.,* 1997).

The ability of KAr to mediate both ionotropic and metabotropic actions may help to explain the proposed ability to facilitate transmitter release at low concentrations, but depress release at higher concentrations at the same synapse. This so called bidirectional regulation has been shown to occur at both glutamate (Schmitz *et al.,* 2001; Lauri *et al.,* 2006) and GABA synapses (Jiang *et al.,* 2001; Braga *et al.,* 2003). In addition, Schmitz *et al*. (2000; 2001) have provided evidence that KAr are present at mossy fibre synapses and both facilitate and inhibit GABA release depending on the duration of the train. This effect, not evident in autoreceptors, has been suggested to be due to different subunit composition (Contractor *et al.,* 2003), but could also be explained by the simultaneous metabotropic and ionotropic modes of action.

1.5.7. KAr in synchronisation of neuronal networks

There is increasing evidence that KAr may be involved in normal and pathological synchronisation of neuronal networks. KAr have been implicated in several types of oscillation, including reducing the frequency of theta oscillations in the hippocampus (Huxter *et al.,* 2007). Cunningham *et al.* (2003) showed that nanomolar doses of both KA and domoic acid (the potent KAr agonist) induced gamma oscillations in the EC in the absence of input from the hippocampus. In a more recent paper, GluR5-containing homomeric KAr have been shown to have a role in the maintenance of these KA-driven gamma oscillations, as UBP 302 and NS 3763 (the homomeric GluR5 selective KAr antagonist; Christensen *et al*., 2004) reduced peak amplitude and spectral power of the oscillations (Stanger *et al., 2008*). In addition, UBP 302 partially inhibited the generation of the oscillations, suggesting that GluR5 containing receptors are at least partly responsible for their induction. It is also interesting to note that gamma oscillations have been shown to occur in superficial layers of the EC in rats who show neuronal loss in layer III of the EC following KA induction of chronic epilepsy, but not in control (Tolner *et al.,* 2005).

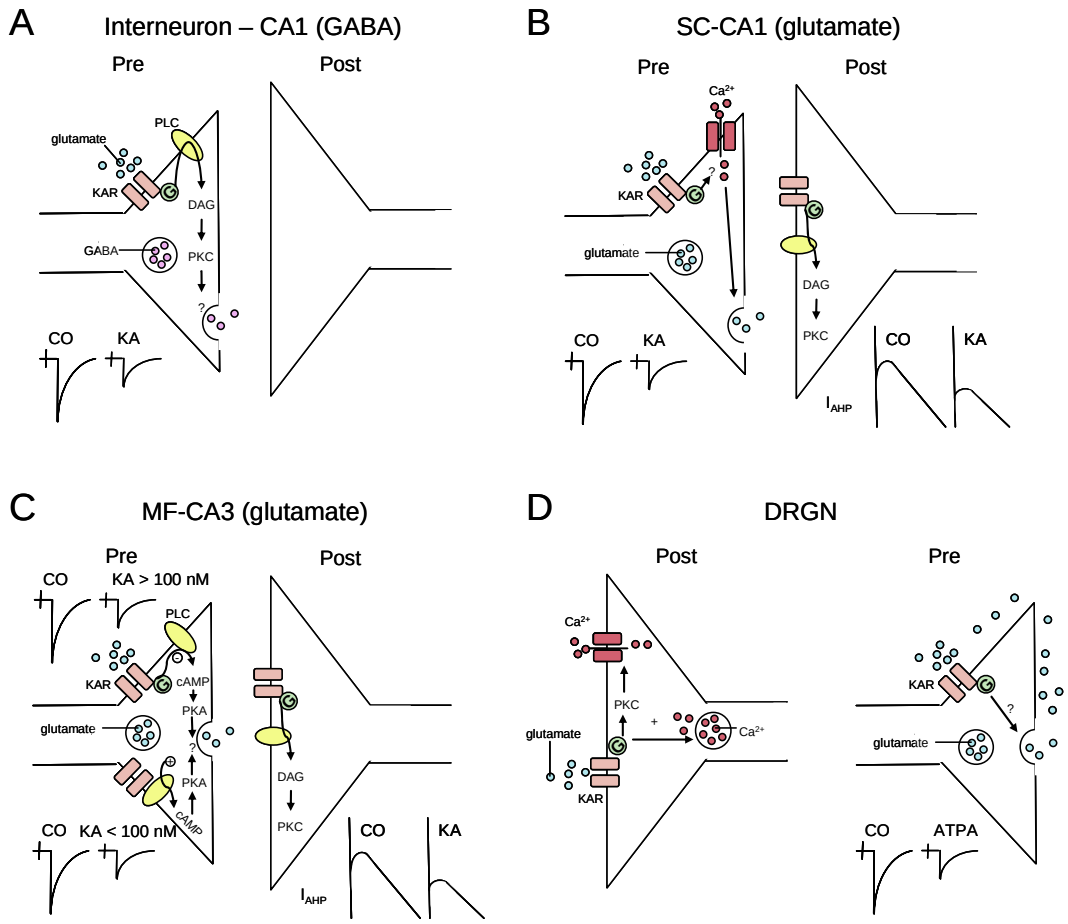


Figure 1.6. Metabotropic actions of KAr. *A: Presynaptic KAr activation enhances GABA release from interneurone terminals onto CA1 pyramidal cell dendrites via a G protein coupled to PLC, which generates second-messenger DAG activating PKC (target unknown)B: Activation of presynaptic KAr on glutamatergic SC terminals decreases glutamate release onto CA1 neurone via presynaptic G-protein activation and regulation of voltage-dependent Ca^{2+} entry. Activation of postsynaptic KAr on CA1 pyramidal cells effects decrease of a Ca^{2+}-activated K^{+} current via a G-protein-mediated activation of PLC and downstream PKC. PKA and mitogen-activated protein kinases are also involved. C: KAr produce a bimodal effect on glutamate release from MFs depending on the agonist concentration;, [KA] > 100 nM decreases glutamate release following activation of a G protein and the modulation of AC and PKA activity, [*KA*] < 100 nM facilitates glutamate release following activation of AC and PKA. Postsynaptic KAr on CA3 pyramidal cells decrease I_{AHP} current via G-protein, PLC and PKC activation. D: Activation of postsynaptic KAr inhibits voltage-dependent Ca^{2+} channels through a G-protein- and PKC-dependent mechanism. At DRGN–dorsal horn neurone synapses, activation of presynaptic KAr by ATPA decreases glutamate release in a potentially G-protein-dependent manner.*

1.5.7.1. Slow wave oscillations

SWO are rhythmically repeating, large amplitude events, which occur at a frequency of less than 1 Hz and are a prominent feature of the EEG during sleep in mammals. They are very similar between species (Zhu *et al.,* 2006) and, thus, are thought to have a relatively conserved function. The intracellular correlate in single neurones is

characterised by periods of irregular tonic firing and a membrane potential fluctuating around -60 mV (an 'up' state) interspersed by periods of hyperpolarisation (a 'down' state). They are often referred to as up-states, and occur in tandem with the SWO of the EEG. These were initially found in the thalamus, in both the thalamo-cortical and thalamic reticular nucleus (Steriade *et al.,* 1993), where they are intrinsic (i.e. require no network input), and can be initiated by stimulating cortico-fugal fibres or application of mGlur agonists. SWO can also be generated in cortical neurones by decreasing Ca^{2+} concentrations, although, in contrast to thalamic neurones, they do require network input (Sanchez-Vives and McCormick, 2000).

The 'up' state component is extremely similar in dynamics, almost indistinguishable, to the active or waking state. This similarity is easily observed both in EEG and extracellular recordings (for review see Destexhe *et al.*, 2007), and has been confirmed by comparing membrane potential dynamics of excitatory and inhibitory conductances using intracellular recordings by Steriade *et al.* (2001) and indirectly through modelling studies (Alvarez and Destexhe, 2004). The relationship between the 'up' state and the waking state has been interpreted as being important for the transfer of memory traces from the hippocampus to the neocortex (Destexhe *et al.*, 2007). This theory is supported by studies in humans, which show that increases in average power density of SWO after a specific learning task are associated with an increased task performance (Huber *et al.,* 2004). Additionally, artificially boosting slow oscillations by transcranial application of oscillating fields increases retention of declarative memories.

1.5.7.2. SWO in the parahippocampal region

An oscillation of less than 1 Hz has been shown to occur in the hippocampus in slow wave sleep and under anaesthetic, which corresponds with the neocortical SWO (Wolansky *et al.,* 2006). Not surprisingly, taking into account the critical positioning of the EC between the neocortex and the hippocampus, they also found that it correlated with slow oscillatory field and multiunit activity in the superficial layers of the EC. This indicated that the hippocampal SWO may be coordinated with SWO from the neocortex via input from the TA pathway. This was confirmed by Isomura *et al.* (2006) who found that neocortical SWO engages neurones in prefrontal, somatosensory, entorhinal, subicular cortices and DG into synchronous transitions

between up and down states and affects the CA1 and CA3 regions of the hippocampus.

This work has instigated the investigation into SWO in the EC. Spontaneous SWO were observed in layer II neurones in EC slices obtained from neonate (P9-13) rats, where they were shown to be NMDAr-dependent (Jones and Heinemann, 1989). Similar events have also been induced in adult rat EC slices by a moderate reduction in $[Mg^{2+}]_o$ (from "normal" 2mM to 1.25 mM). In these experiments, SWO were abolished by the GluR5 antagonist, UBP 302 (Cunningham *et al.,* 2006). However, although these experiments suggested that NMDAr were not involved (Cunningham *et al.,* 2006), further work in this laboratory suggests that these SWO can be reduced or abolished by either the KAr antagonist or the competitive NMDAr antagonist 2-AP5 (Greenhill and Jones, unpublished data). Thus, both KAr and NMDAr appear to contribute to SWO in the EC.

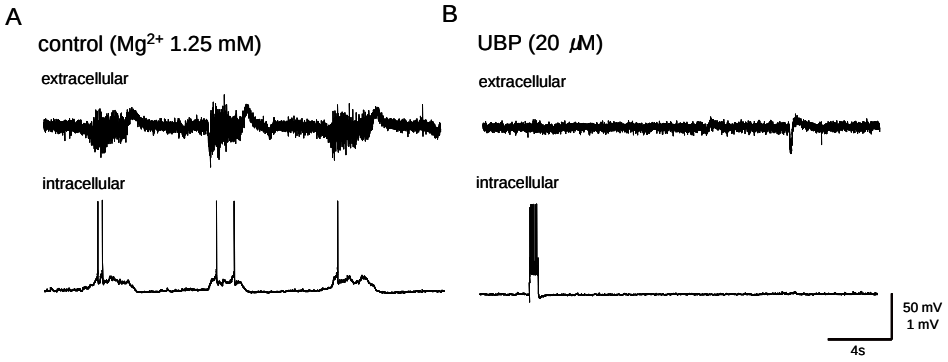


Figure 1.7. SWO in the EC. *A: Concurrent recordings of the LIII field potential and a LIII pyramidal neurone showing the spontaneous generation of SWO in layer III when extracellular magnesium is reduced. B: Application of the GluR5 receptor antagonist UBP 302 (20 μM) abolished the SWO. At the asterisk, a depolarising current step demonstrates the continued ability to evoke neuronal responses.*

1.5.7.3. Pathophysiological roles of KAr

Evidence supporting a role for KAr in various pathological conditions has begun to accumulate. The GluR6 allele has been associated with early onset Huntington's disease (Macdonald *et al.,* 1999) and GluR6 mediated excitotoxicity has been implicated in the pathology of the disease (Rubensztein *et al.,* 1997). The GluR5 gene is located on chromosome 21q22.1 (Barbon and Barlati, 2000) which is a region triplicated in Down's syndrome. A disruption in GluR6 transmission has also been

identified in both autism (Jamain *et al.,* 2002) and schizophrenia (Bah *et al.,* 2004). Also, in schizophrenia, a reduction in both GluR6 and KA2 mRNA in the hippocampus has been demonstrated (Porter *et al.,* 1997).

A large majority of the evidence has been for the possible involvement of KAr in epilepsy. It has been known for many years that systemic or local administration of KA produces seizures and patterns of neuronal damage that are closely reminiscent to those observed in patients with epilepsy (Ben-Ari, 1985). Thus, KA administration has become a common method to induce an animal model of the disease. But is it the KAr itself which is mediating these pathological actions of KA? Several authors have provided evidence in agreement with this hypothesis. GluR6 knockouts decrease susceptibility to KA induced epilepsy (Mulle *et al.,* 1998), although this was more pronounced for lower concentrations of KA. KAr mRNA has been found to be altered in temporal lobe epilepsy patients (Mathern *et al.,* 1998) and the GluR5 allele (Sander *et al.,* 1997) but not the GluR6 allele (Sander *et al.,* 1995) increases susceptibility to the development of juvenile absence seizures. These observations indicate that the KAr plays an important role not just in KA induced epilepsy but also in the epileptic disorder in general.

Since KAr appear to mediate a decrease in GABA release in various brain regions (Rodriguez-Moreno *et al.,* 1997; Braga *et al.,* 2003), some authors have postulated that this may be responsible for the acute epileptogenic activity of KA, giving rise to the idea of KAr antagonists being a possible future therapy for epilepsy. There has been some evidence to support this theory. For example, Smolders *et al.* (2002) showed that selective GluR5 antagonists (LY 377770 and LY 382884) prevent the development of epileptiform activity induced by pilocarpine in hippocampal slices and also prevent limbic seizures provoked by both chemical and electrical stimulation in awake animals. In addition, the anticonvulsant, topiramate, has been shown to protect against seizures caused by a GluR5 specific KAr agonist (Kaminski *et al.,* 2004).

However, this does not reconcile well with the ability of KAr to increase GABA release in some brain regions (Cossart *et al.,* 2001) or indeed take into account the varied actions of KAr at glutamatergic synapses. It may be that the traditional assumption that a collapse in inhibition is responsible for epileptic activity is incorrect. It is possible that an increase in GABA-mediated inhibition may lead to an

enhancement of hypersynchronous gamma activity and 'over binding' thus precipitating epileptogenesis (Medvedev, 2002; see Section 1.3.2.). In addition, it has also been recently suggested that an accumulation of chloride may lead to a change in the reversal potential of GABAergic currents causing a shift from inhibition to excitation, as is seen in development (Cossart *et al*., 2005). Therefore, it is possible that an decrease in GABA release could prove to be beneficial in epilepsy.

Whilst a direct activation of KAr, whether to decrease or increase inhibition, may account for the acute actions of KA it does not explain the chronic epilepsy that is generated long after the KA treatment. However, recent evidence has also implicated a more long-term involvement of KAr in TLE, specifically in the sprouting of mossy fibres (Epsztein *et al.* 2005), a well known feature of the disease (Sutula *et al.,* 1989). Mossy fibre axons reroute following epileptic cell death to synapse with granule cell dendrites in the inner molecular layer of the DG creating a hyper-excitable circuit. Epsztein *et al.,* (2005) have shown that in pilocarpine treated rats a prominent KAr mediated current (blocked by SYM 2081) was present in dentate granule cells that was not seen in control and also that SYM 2081 also acted to reduce the epileptiform activity in the presence of high K^+ artificial cerebrospinal fluid (aCSF).

Collectively these results show that KAr are involved in several forms of oscillatory behaviour including oscillations occurring as a result of epileptogenesis and therefore possibly play a role in the synchronisation of cortical networks and that adaptive changes in receptor function or expression may be in part responsible for the pathological synchronisation underlying epilepsy.

CHAPTER 2

METHODS

2.1. Introduction

In order to investigate the function of KAr in the EC I have utilised the electrophysiological technique of whole-cell voltage-clamp. In this technique a patch pipette is placed close to the cell body and negative suction is applied to form a high resistance electrical seal. The membrane contained within the tip of the electrode is then ruptured by application of further negative pressure so that the patch pipette then becomes electrically continuous with the inside of the cell. This allows currents to be measured in the whole cell enabling us to monitor the changes caused by the opening and closing of ligand gated ion channels on the postsynaptic membrane. These are excitatory or inhibitory post synaptic currents (E/IPSCs) depending on whether they are mediated by the amino acids glutamate or GABA, respectively.

I have looked at the role of KAr in mediating both pre- and postsynaptic effects of glutamate. Analysis of the effects of the application of various agonists and antagonists for ligand gated ion channels, has allowed me to gain information about the presence and function of these receptors at postsynaptic sites on glutamatergic neurones, and on presynaptic terminals of both glutamate and GABA neurones. To look at the postsynaptic contribution of KAr to excitatory responses, I evoked EPSCs (eEPSCs) by stimulating presynaptic pathways electrically using an external electrode placed on the surface of the slice, and used antagonists of other receptors to pharmacologically isolate the KAr component.

In order to study presynaptic KAr, we would ideally like to record directly from the presynaptic terminals themselves. However, with a few exceptions (e.g. Calyx of Held terminals, CA3 mossy fibre boutons), this is not feasible as terminals in cortical areas like the EC are too small to be visualised and patched directly. Presynaptic effects of KAr activation were therefore inferred from two approaches. Firstly, from alterations in eEPSCs and evoked IPSCs (eIPSCs) induced by agonists and antagonists. Secondly by monitoring changes in frequency of postsynaptic currents, resulting from the spontaneous release of glutamate or GABA from presynaptic terminals, which results in the spontaneous openings of postsynaptic channels. Thus the sEPSCs and sIPSCs can be used as a reporter or recorder of presynaptic transmitter release, which can give us information regarding the mechanisms that control the release of transmitter, such as the presence of presynaptic KAr.

2.2. Slice preparation

Combined entorhinal-hippocampus slices were prepared from male juvenile Wistar rats (21-35 days old; 45-70 g), as described previously (Jones & Heinemann, 1988). All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act 1986, European Communities Council Directive 1986 (86/609/EEC) and the University of Bath ethical review document. In initial experiments, rats were first anaesthetised using an i.m. injection of ketamine (120 mg/kg) plus xylazine (8 mg/kg) and then decapitated. In later studies the animals were killed by cervical dislocation followed by decapitation without prior anaesthetic. Whilst the former approach produced better quality, longer lasting slices, there was no apparent difference in the results gained with either approach, so no distinction has been made between them.

Following decapitation, the brain was rapidly removed and immersed in oxygenated aCSF (see below for composition) chilled to around 4 °C. The cerebellum was then removed and the brain bisected along the midline. A small slab of tissue was removed from the dorsal surface of the brain and this provided a flat surface on which the remainder of the brain was fixed, ventral surface upwards, to a Teflon slicing block using cyano-acrylate glue. The block plus brain was then immersed in the slicing chamber of a Campden Vibroslice (Campden Instruments), which was filled with chilled aCSF oxygenated with carbogen gas (95 % O_2 / 5 % CO_2). Slices were then cut through the brain from a caudal direction using a vibrating ceramic blade moved horizontally through the tissue at a slow speed. The frequency of vibration (intermediate) and speed (approximately 1.5 mm/min) at which the slices were cut were both chosen according to previous experience in the laboratory as providing the best structural preservation and viability of tissue as possible. Slices were discarded until the most ventral portion of the hippocampus and the rhinal fissure could be seen. From this point, slices (4-6; 350 μM thick) from each hemisphere, and containing the hippocampus, dentate gyrus, the subicular areas, and the entorhinal and perirhinal cortices, were dissected away from the rest of the brain (Figure 2.1).

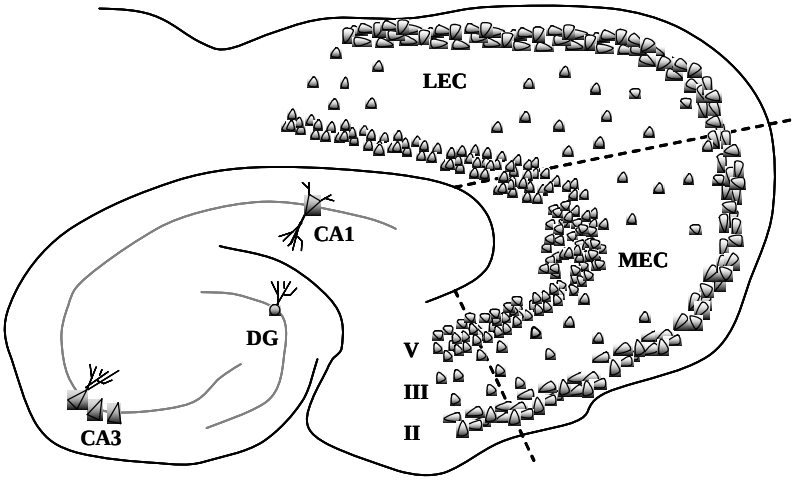


Figure 2.1. Schematic diagram of the combined entorhinal-hippocampal slice *showing the location of CA1 and CA3 pyramidal cells, dentate gyrus granule cells and the medial and lateral entorhinal cortex.*

These slices were then removed as they were cut and transferred to storage in a BSC-PC chamber (Warner Instruments; figure 2:2) containing aCSF at room temperature. This allows up to 12 brain slices to be safely stored *in vitro* while maintaining excellent slice viability. Carbogen gas was bubbled into the chamber, and this initiates a circular flow of oxygen enriched aCSF, which continuously permeates the slices allowing them to remain viable for up to 8 hours while awaiting transfer to the recording chamber.

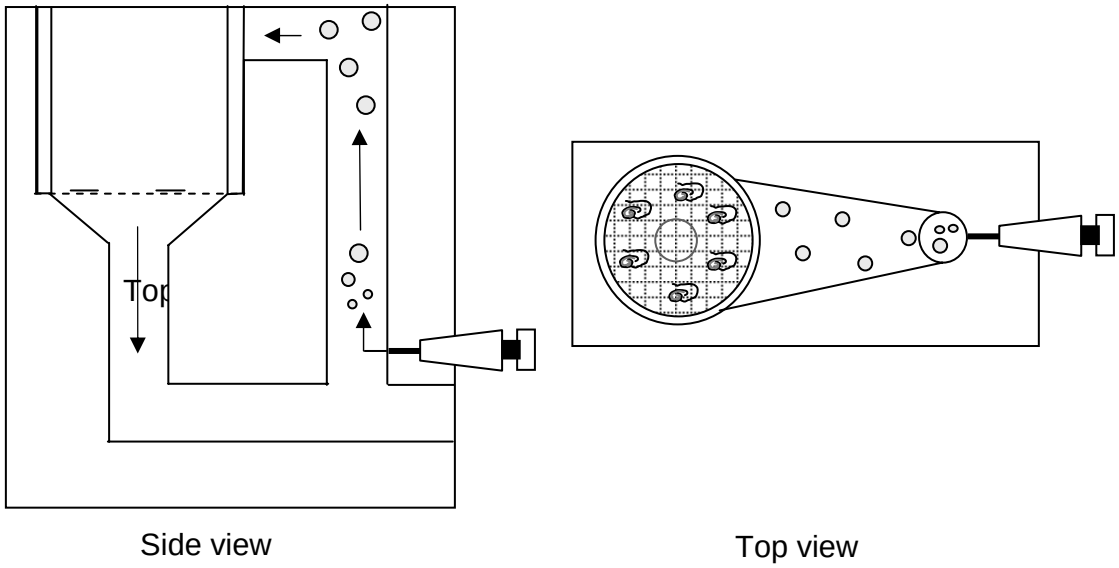


Figure 2.2. Schematic diagram of the BSC-PC slice holding chamber.

Slices were left to equilibrate in storage for at least one hour before being transferred individually, when required, to a recording chamber mounted on the stage of an upright Olympus BX50WI microscope. The chamber was perfused (2ml/min) with oxygenated aCSF and temperature was set at 31 ± 1 ºC using an inline heater (SH-27B, Warner Instrument Corporation) and a thermostatically controlled feedback power supply (TC-324, Warner Instruments). Slices were allowed to further equilibrate in the recording chamber for 30 minutes before recording was attempted.

Slices were visualised using a CCD infrared video camera (KP-M1E/K, Hitachi Kokusai Electric Inc.). All neurones for this study were recorded in the medial EC from layer III (unless otherwise indicated). Cells were selected based upon their appearance as healthy, undamaged neurones with clear pyramidal morphology. However, although the neurones studied were presumed to be glutamatergic pyramidal neurones, no attempt was made to retrospectively confirm this using dye injection techniques and post-hoc morphological identification.

2.3. aCSF

The standard artificial cerebrospinal fluid used for dissection, slicing and recording contained (in mM) NaCl (126), KCl (3) NaH_2PO_4 (1.4), $NaHCO_3$ (19), $MgSO_4$ (2), $CaCl_2$ (2), and D-glucose (10). The dissection and slicing solution also contained ketamine (an NMDA receptor antagonist which at higher doses may also bind to opioid receptors that induces dissociative anaesthesia; 150 μM), indomethecin (a non-steroidal anti-inflammatory drug which inhibits the production of prostaglandins; 45 μM) and either n-acetyl-l-cysteine (NAC; derivative of the amino acid L-cysteine, and is a precursor in the formation of the antioxidant glutathione in the body and is able to reduce free radicals; 6 μM) or uric acid (Uric acid is the final product of purine metabolism in humans and there is increasing evidence showing that uric acid has an important role in vivo as an antioxidant (for review see Glantzounis *et al*., 2005); 300 μM), all of which we have determined from experience in this lab can increase the survival and viability of neurons. In the experiments where reduced $[Mg^{2+}]_o$ was employed, the storage and recording aCSF was modified to contain KCl at 3.75 mM and $MgSO_4$ at 1.25 mM.

2.4. Recording

Patch pipettes (open tip resistance 1–4 MΩ) were pulled from borosilicate glass (PG120T-10, 1.2 mm O.D. x 0.93 mm I.D., Harvard Apparatus) on a Flaming/Brown microelectrode puller. Pipette tips were coated with a layer of surf board wax (Mr. Zog's Sex Wax) to minimise pipette-bath capacitance and electrical noise. Whole-cell voltage-clamp recordings were made of the EC using an Axopatch 200B amplifier.

For EPSC recordings patch pipettes were filled with a Cs-gluconate-based patch solution containing the following (in mM): D-gluconate (100). HEPES (40), QX-314 (1), EGTA (0.6), NaCl (4), $MgCl_2$ (5), tetraethylammonium-Cl (1), ATP-Na (4), GTP-Na (0.3). The solution was adjusted to 275 mOsm by dilution and set to pH 7.3 with CsOH. Using this patch solution and with membrane potential nominally voltage clamped at –60 mV, neurones continuously displayed sEPSCs which were primarily mediated by sodium ion entry through AMPA receptors activated by spontaneous glutamate release from presynaptic terminals (*cf*. Berretta and Jones, 1996a).

For inhibitory recordings patch pipettes were filled with a Cs-based patch solution containing the following (in mM): CsCl (100), HEPES (40), QX-314 (1), EGTA (0.6) $MgCl_2$ (5), tetraethylammonium-Cl (10), ATP-Na (4), GTP-Na (0.3). The solution was again adjusted to 275 mOsm by dilution and set to pH 7.3 with CsOH. Using this patch solution and with membrane potential again voltage clamped at –60 mV, neurones continuously displayed sIPSCs which were mediated by chloride (Cl^-) ion entry through $GABA_A$r activated by spontaneous GABA release from presynaptic terminals (*cf.* Woodhall *et al*., 2005).

2.5. Evoked responses

To investigate postsynaptic KAr at glutamate synapses and presynaptic KAr at both glutamate and GABA synapses we examined eEPSCs and eIPSCs. These studies involved electrically activating the presynaptic neurones/axons via a bipolar platinum electrode. Stimulating electrodes were positioned with the tips on the surface of the slice in layer V of the lateral EC close to the recording pipette in layer III (see figure 2.3). Stimulus pulse duration was 0.05 ms with intensity (constant voltage: 1 - 10 mV) and stimuli controlled using a either a Grass S48 Stimulator or a Master-8 pulse

generator (A.M.P.I. Ltd, Jerusalem) coupled to a DS2 stimulus isolation unit (Digitimer Ltd.). The stimulus intensity was set to give an initial evoked response that was approximately 80% of maximal. Stimulation was delivered in trains of varying frequencies. Train parameters were set using the Grass S48 stimulator or Master 8. A number of different stimulation protocols were used and these will be described in detail in the relevant chapters.

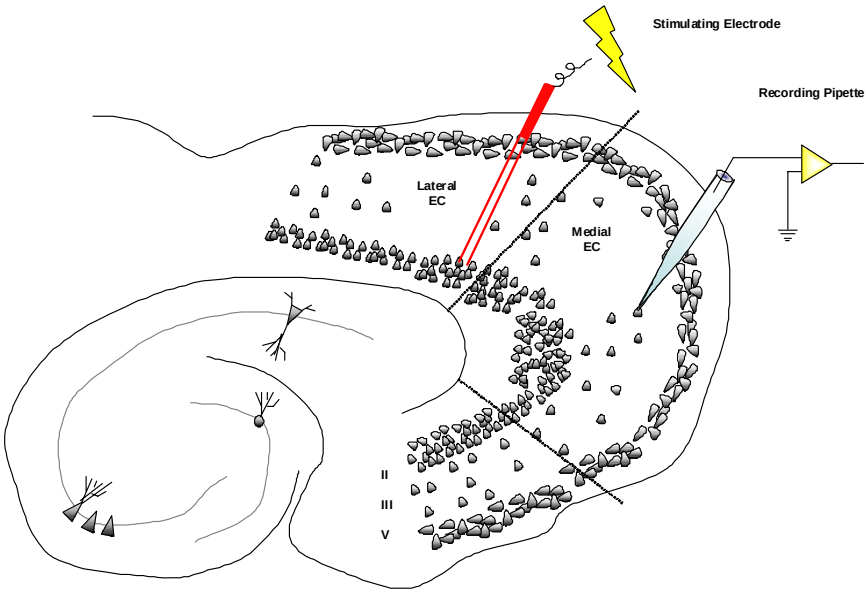


Figure 2.3: Diagrammatic representation of the combined EC-hippocampal brain slice showing the positioning of the bipolar stimulating electrodes used elicit eI/EPSCs in layer III of the mEC.

2.6. Activity-independent synaptic events.

Spontaneous transmitter release is comprised of two components. Both glutamate and GABA can be released as a consequence of action potentials invading the presynaptic terminal, and subsequent Ca^{2+}-influx through voltage-gated Ca^{2+}-channels. The second component is action potential-independent, reflects random monoquantal release of transmitter, and is independent of extracellular Ca^{2+}. Blocking voltage-gated Na^{+} channels and, thus, action potentials can abolish activity-dependent release. This allows isolation of the activity-independent events, so called "miniature" or mEPSCs and mIPSCs. These have been examined in a number of studies in this thesis by blocking presynaptic action potentials by bath perfusion with TTX (1 μM).

2.7. Data acquisition and analysis

Whole-cell voltage clamp recordings were filtered at 2 - 5 kHz and digitised (Digidata 1200B, Axon Instruments) at 20 - 50 kHz. Data were recorded to computer hard disk using Axoscope software. Series resistance compensation was not used, but access resistance (10–30 MΩ) was monitored at regular intervals throughout each recording, and cells were discarded from analysis if it changed by more than ± 10 %. Liquid junction potentials (LJP) were estimated using the calculator of pClamp 8 software. With the patch solution employed for EPSC recordings this was +12.6 mV, and with IPSC patch solution it was +10.13 mV. The junction potentials were not routinely compensated for in the holding potentials, but were in cases where current-voltage relationships were examined.

Recordings were saved on-line to computer hard disc using AxoScope software (Axon Instruments). All data (spontaneous and evoked responses) were analysed off-line using Minianalysis software (Synaptosoft, Decatur). Analysis of evoked PSCs largely consisted of measuring peak amplitudes. However, in some experiments rise and total decay times of evoked potentials were also examined.

Spontaneous events (either sEPSC/IPSCs or mEPSC/IPSCs) were detected automatically using a threshold-crossing algorithm and their interevent interval (IEI, inversely proportional to frequency), amplitude, rise (10-90%) and total decay times determined. Threshold was determined in order to detect the smallest spontaneous events above baseline noise and was maintained at a constant level, individually set for each neurone. When comparing s/mEPSCs or s/mIPSCs a minimum of two hundred events during a continuous recording period was sampled in control conditions and compared to the same number of events recorded in the drug perfusion periods. As noted, events were detected and marked automatically, but each event file was manually inspected in order to discard any events that were clearly non-biological from subsequent analysis. To compare pooled data under control and drug conditions, we determined mean values for event parameters in each cell and calculated the mean values for the population, using a paired t-test or one way analysis of variance (ANOVA) with Bonferroni Test, where appropriate, for statistical comparison. In addition, cumulative probability distributions of IEI were also determined and statistically compared using the non-parametric Kolmogorov-Smirnoff (KS) test.

In a number of studies of evoked PSCs we also conducted a 'before and after' analysis. In these studies, the number of sEPSCs or IPSCs in a 1s time period immediately before a stimulus train was determined and compared (together with event amplitude) to the number in the 1s immediately after the train. Event number and amplitude were statistically compared using a paired t-test.

For evoked EPSCs the mean amplitudes of the events in at least three consecutive trains were calculated for each condition (control *vs* drug).

All error values stated refer to the standard error of the mean.

2.8. Pharmacological studies

All drugs were applied by inclusion in the bath perfusion medium. Specific application protocols are given in the relevant data chapters. Control recordings were conducted for periods of at least 10 minutes or more to ensure stability. Data sampling in the presence of drugs was conducted at least 10 minutes after the drug had reached the recording chamber. Bath volume was maintained at approximately 700 µl. Dead-time after changing perfusion solutions was approximately 40 seconds, so with the perfusion rate employed exchange of drug solutions in the recording chamber was quite rapid. After completion of a successful pharmacological protocol on a recorded neurone, the slice was discarded and each new study performed on a naive slice transferred from the holding chamber.

2.9. Drugs used.

Drugs were made up as stock solutions (indicated below), aliquoted and stored at -20°C. They were thawed immediately before use and diluted to the desired concentration in aCSF.

2-AP5 (D-2-Amino-5-phosphonopentanoic acid), Tocris, UK. For stock solution dissolved in water.

ATPA ((RS)-2-Amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid), Tocris, UK. For stock solution dissolved in water.

Bicuculline methiodide ([R-(R*,S*)]-5-(6,8-dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetra-hydro-6,6-dimethyl-1,3-dioxolo[4,5-g]isoquinolinium iodide), Tocris, UK. For stock solution dissolved in water.

CGP 55845A ((2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydro xypropyl](phenylmethyl)phosphinic acid), Tocris, UK. For stock solution dissolved in DMSO.

CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione), Tocris, UK. For stock solution dissolved in DMSO.

GYKI 53665 (1-(4-aminophenyl)-3-methylcarbamoyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine) a kind gift from Dr Dick Evans, formerly of Bristol University. For stock solution dissolved in DMSO.

MK 801 (Dizocilpine maleate), Tocris, UK. Dissolved in patch solution.

NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), Tocris, UK. For stock solution dissolved in DMSO.

NS 3763 (4,6-Bis(benzoylamino)-1,3-benzenedicarboxylic acid), Tocris, UK. For stock solution dissolved in DMSO.

PDC (L-trans-Pyrrolidine-2,4-dicarboxylic acid), Tocris, UK. For stock solution dissolved in water.

SR 95531/Gabazine (6-Imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide), Tocris, UK. For stock solution dissolved in water.

SYM 2206 ((±)-4-(4-Aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphthalazine), Tocris, UK. For stock solution dissolved in DMSO.

TTX (Tetrodotoxin) with citrate, Alomone Labs., Israel. For stock solution dissolved in water.

UBP 302 ((S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrimidine-2,4-dione), Tocris, UK, and also a gift from Dr Dave Jane, University of Bristol. For stock solution dissolved in 1 eq. NaOH.

CHAPTER 3

SPONTANEOUS EXCITATION AND INHIBITION IN LAYER III PRINCIPAL NEURONES

3.1. Introduction

As noted in Chapter 1, increasing attention is being focused on layer III of the EC, both in terms of physiological function (e.g. Erchova *et al*., 2004; Cunningham *et al*., 2003; 2006) and pathological dysfunction (e.g. Du *et al*., 1993; 1995; Gloveli *et al*., 2003). In contrast to neurones in other layers of the EC, less is known about basic properties of layer III neurones. Our laboratory has previously described in detail the characteristics the sEPSCs and sIPSCs in layers II and V of the rat EC *in vitro* (Berretta and Jones, 1996a; Woodhall *et al*., 2005) and shown clear differences between the two layers. During the course of the work described in this thesis I have made many recordings of spontaneous synaptic events in layer III neurones. Therefore, the aim of this chapter is to describe the properties of background spontaneous excitatory and inhibitory currents in layer III, to provide a more complete picture of layer III neurones and enable a comparison to what has been previously published for deeper and more superficial layers.

Berretta and Jones (1996a) compared sEPSCs in layers II and V in the rat EC using whole cell patch clamp. They showed that the frequency of sEPSCs was similar in the two layers, but the events in layer V had a slightly larger mean amplitude, faster rise time, and were faster to decay. They attributed this to the presence of a population of larger events in the layer V neurones that were not present in layer II. They also noted that the frequency of sEPSCs in both layers was reduced by TTX to a similar extent (15-20%). However, the amplitude distribution was unchanged in layer II, whereas in layer V TTX blocked most of the larger amplitude sEPSCs. In terms of the pharmacology sEPSCs, most were abolished by addition of NBQX (or CNQX), indicating that the majority of sEPSCs in these layers were mediated by AMPAr. There was a small proportion of events left in NBQX that had a slower time course and were abolished by 2-AP5, indicating they were mediated by NMDAr. Although very infrequent, NMDAr-mediated events were more evident in layer V than layer II.

Woodhall *et al*. (2005) compared the properties of sIPSCs in layers II and V in the rat EC. They showed that the sIPSCs were apparently exclusively mediated by $GABA_A$r, as were abolished by gabazine. They had similar average amplitude and decay times but 10-90% rise times were slightly faster in layer V. However, the major difference between the layers was in frequency. The frequency of events in layer II neurones was

4-5 times greater than that seen in layer V. When recorded in the presence of TTX, the frequency of events was more than halved in layer V, whereas there was only a slight reduction in layer II, indicating that the large majority of events in layer II are action potential-independent. High frequency bursts of sIPSCs were seen in both layers but they occurred much more frequently in layer II than in layer V. Although, there was little difference in average decay or rise times in layers II or V, detailed analysis of the decay kinetics of individual sIPSCs indicated that they could be subdivided according to the relative decay and or rise times. It was suggested that this could indicate inputs from different subtypes of interneurones and/or inputs at different somatodendritic locations.

Thus, this chapter aims to extend our knowledge of the properties of neurones in the EC by providing a characterisation of sEPSCs and sIPSCs in layer III and to compare these characteristics to those previously reported in other principal neurones of the EC.

3.2. Methods

In all experiments in this chapter, brain slices were prepared from juvenile animals aged from 3-5 weeks old (45 - 70 g). sEPSCs and sIPSCs were recorded from neurones in layer III of the medial division of the EC. In all recordings examining IPSCs, 2-AP5 and GYKI 53665 were included in the bath to block NMDAr and AMPAr respectively.

Although recordings have been made from over 250 neurones, analysis in this chapter was limited to selected populations of high quality recordings i.e. cells with good access resistance (<15 MΩ; which was calculated from a 10 mV voltage step of 10 ms at a holding potential of -60 mV) which remains stable for the duration of the recording and high input resistance (>200 MΩ; calculated from the current shift at the end of the 10 ms pulse). Recordings were also only included in the analysis for this chapter if the holding current was less than 100 pA and if the holding current was remained unchanged throughout the experiment. For analysis, two hundred events during a continuous recording period were sampled for each neurone under each condition. Mean values for IEI, amplitude, rise and decay times were determined for each neurone from the 200 events. Pooled data were compared by averaging means

from the population and a Student's t-test was used for comparison. Pooled data were also compared by constructing cumulative probability distributions and non-parametric statistical analysis with the KS test.

3.3. Results

3.3.1. Characterisation of sEPSCs.

3.3.1.1. Amplitude and frequency of sEPSCs

The detailed analysis of sEPSC frequency and amplitude was conducted on representative whole-cell recordings made from a total of 15 neurones in layer III of the medial EC. At a holding potential of -60mV, neurones in layer III of the EC exhibited EPSCs as spontaneously occurring inward currents (Figure 3.1A). The mean IEI of sEPSCs was 217 ± 32 ms, which corresponds to a mean sEPSC frequency in the 15 neurones of 4.6 ± 1.0 Hz. This is considerably faster than that previously reported for either layer II and V cells (1.4 ± 0.1 Hz and 1.4 ± 0.1 Hz respectively; Beretta and Jones, 1996a; Figure 3.1B). Although sEPSC frequencies vary considerably from cell to cell and study to study, overall, layer III clearly shows a higher frequency of sEPSCs than either its deeper (V) or more superficial (II) counterparts (*cf* studies in Jones and Woodhall, 2005).

The mean amplitude of sEPSCs in these neurones was 13.7 ± 0.9 pA. This is closer to that seen in layer II than layer V (13.24 ± 0.26 pA and 15.67 ± 0.52 pA respectively; Beretta and Jones, 1996a; Figure 3.1D). sEPSCs rarely exceeded 60 pA in amplitude. As in both layers II and V, the distribution of layer III sEPSCs was represented by a histogram with a single peak, skewed toward larger amplitude events. This is shown in Figure 3.1 together with the comparison with previous data from layers II and V.

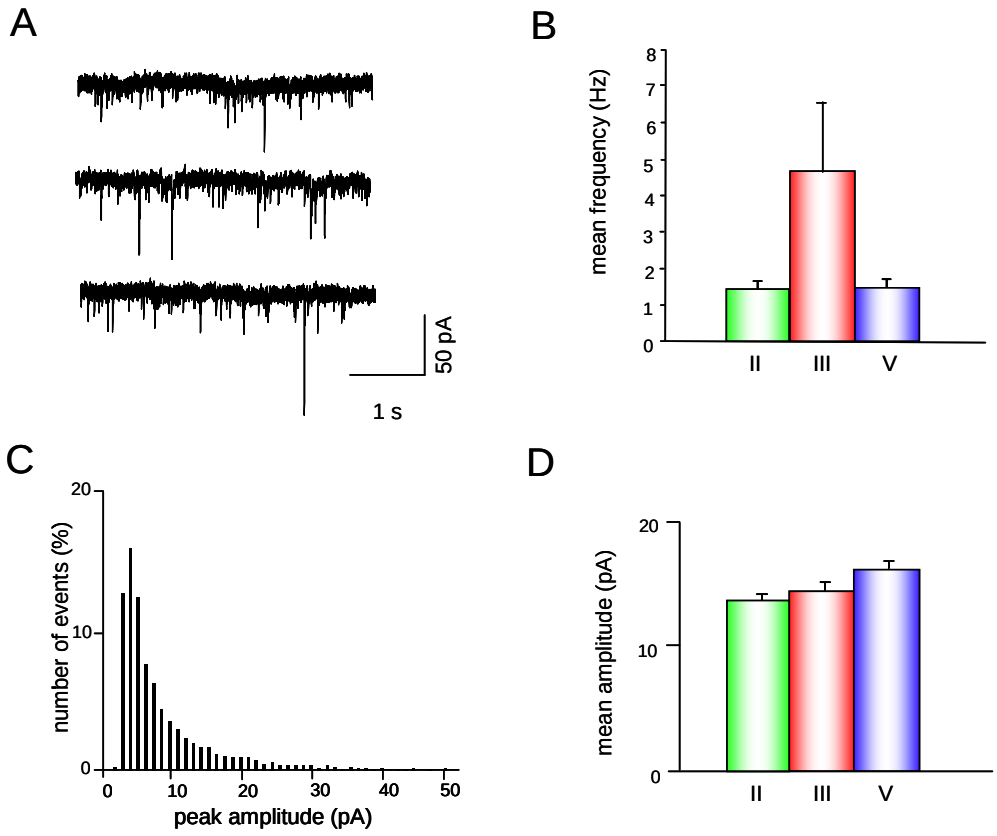


Figure 3.1. Comparison of sEPSC amplitude and frequency in EC neurones. *A: The traces are consecutive voltage clamp recordings from a typical layer III neurone. The downward deflections are sEPSCs. B: The graphs show the mean frequency of sEPSCs in layer III, compared to values for layers II and V (data from Berretta and Jones, 1996a). C: Frequency distribution of sEPSC amplitudes in layer III neurones (bin width = 1). The distribution shows a single peak, skewed toward larger amplitude events. D: Comparison of average sEPSC amplitudes in layer III with layer II and V (data from Berretta and Jones, 1996a).*

3.3.1.2. sEPSC bursts

In their analysis of sEPSCs, Beretta and Jones (1996a) described the presence of prominent bursts in layer V (see Fig 1A Beretta and Jones, 1996a), but these occurred infrequently in layer II. In layer III neurones, burst activity (defined as at least 3 events in a 50 ms time frame) was seen in most neurons (13/15) although it was not particularly frequent (1.8 ± 0.3 bursts per neurone). Examples can be seen in Figure 3.2. Analysis gave an inter-burst interval of 6.4 ± 2.4 s. The mean number of events per burst was 3.4 ± 0.1. Further analysis of these bursts revealed that the IEI of sEPSCs within bursts was 11.1 ± 1.5 ms and the mean duration was 38 ± 3 ms. Unfortunately, such detailed analysis of bursts was not presented by Beretta and Jones (1996a), so it is not possible to make any detailed comparisons. However, as it is likely that bursts in these neurones are driven by pyramidal neurones within the EC

(as excitatory inputs from outside the EC would have been largely removed during slice preparation) the increase propensity for bursting in layer III may be a reflection of high baseline firing rates in principal neurones connected by extensive recurrent excitatory collaterals in this layer compared to layers II and V.

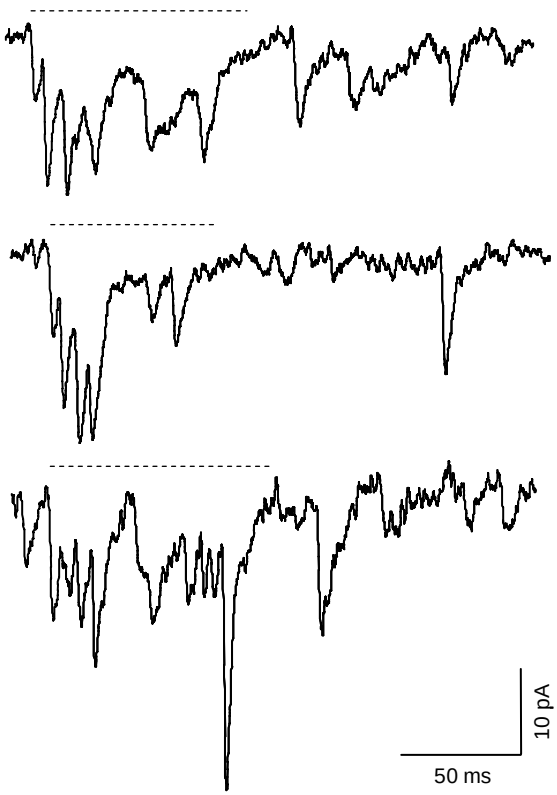


Figure 3.2. Bursts of sEPSCs in layer III neurones. *The traces are voltage clamp recordings showing bursts of sEPSCs (defined as at least three events within a 50 ms time frame) from typical layer III neurones. Bursts are indicated by the dotted line above the trace. Bursting activity was seen in most cells but within cells they were not particularly frequent.*

3.3.1.3. Effect of TTX on EPSCs

Spontaneous transmitter release at synapses occurs in two ways. The first of these is caused by the opening of VGCC following an action potential that invades the terminal. This is often referred to as 'true' spontaneous (action potential-dependent) release. Random, monoquantal release can also occur, and this is held to be independent of action potentials and Ca^{2+} entry into the terminals, so called 'miniature' events. Separation of the two can be achieved by blocking action potentials with TTX.

In 6 layer III neurones, TTX (1 μM) increased the IEI (decreased frequency) of events by more than six-fold from 217.4 ± 32 ms to 1359.6 ± 261 ms (n=6; KS test – $P < 0.0001$; Figure 3.3A,B). This represents a decrease in frequency from 6.5 ± 1.0 Hz to 0.9 ± 0.2 Hz, indicating that a very large majority of sEPSCs in this layer are action potential-dependent. This contrasts with the situation in both layers II and V where application of TTX only caused a 15-20 % reduction in the frequency of sEPSCs (Beretta & Jones, 1996a; Figure 3.4C) showing that the majority of events in these layers are action potential-independent. However, layer III shows some similarity to layer V in that TTX also caused a shift in the cumulative amplitude distribution (KS test – $p < 0.0001$; Figure 3.3D) towards smaller events, suggesting a loss of larger amplitude events, an effect not seen in layer II neurones (Beretta & Jones, 1996a). However, the mean amplitude of layer III sEPSCs remains unaffected (13.8 ± 0.9 pA vs. 12.5 ± 1.1 pA; Figure 3.3E). TTX also had no effect on the mean decay time (from 5.4 ± 0.5 to 4.6 ± 0.4) or 10-90 % rise time (from 1.8 ± 0.1 to 1.8 ± 0.2).

3.3.1.4. Kinetics of sEPSCs

sEPSCs in layer III had a mean 10-90% rise time of 1.8 ± 0.1 ms and a decay time of 5.4 ± 0.5 ms (n = 15). This is very similar to kinetic properties previously found for layer V (rise time = 1.9 ± 0.1 ms and decay time 5.7 ± 0.3 ms; Beretta and Jones, 1996a). sEPSCs in layer II display comparatively slower rise and decay kinetics (rise time = 2.5 ± 0.1 ms and decay time 8.0 ± 0.4 ms; Beretta and Jones, 1996a; Figure 3.4). Differences in kinetics may be due to differences in electrotonic properties of neuronal subtypes. Previously, electrotonic lengths have been estimated during whole-cell voltage-clamp recordings (Berretta and Jones, 1996a; Jones, unpublished). Electrotonic lengths in layer V and III were found to be similar, whereas layer II neurons were less compact. A similarity in electronic length between layer V and layer III neurones may reflect the similar sEPSC kinetics. It has been suggested by several studies that as a result of electrotonic filtering (the distortion of synaptic currents as a function of distance from the soma – Spruston *et al.,* 1993), correlation of kinetic parameters may enable the identification of postsynaptic events arising from different cell surface domains (McBain and Dingledine. 1992). In view of this correlations were sought between rise time and decay time in layer III neurones, and also between rise time and amplitude for the pooled data of both sEPSCs and mESPCs. Figure 3.4D & E clearly shows that there were no significant correlations

for any of these parameters either for spontaneous or miniature EPSCs with r^2 values all less than 0.1. This is perhaps unsurprising as Cs was included in the patch solution which would decrease space clamp errors by blocking potassium channels and therefore differences in kinetic parameters of events arising from various distances from the soma would be largely unapparent.

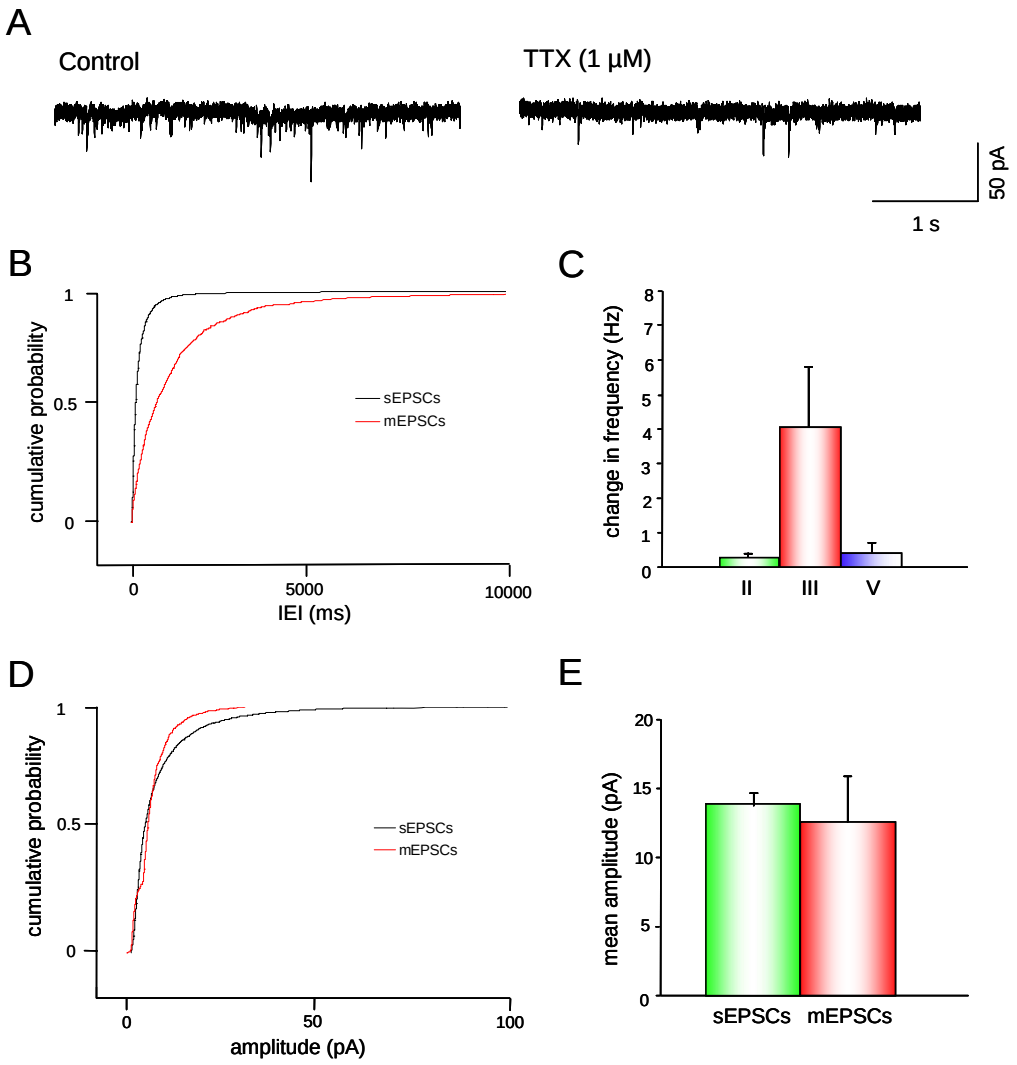


Figure 3.3. Comparison of sEPSCs and mEPSCs in EC neurones. *A: The traces are consecutive voltage clamp recordings from a typical layer III neurone in the absence and presence of TTX (1 µM). B: Cumulative probability graph of IEI of sEPSCs compared to mEPSCs from layer III neurones (n=6). C: Graph of change in frequency after addition of TTX (frequency of sEPSC-frequency of mEPSC) in layer III neurones, compared to layer II and V neurones (data from Berretta and Jones, 1996a). D: Cumulative probability graph of amplitude (n=6) of mEPSC and sEPSC amplitudes in layer III neurones. E: Comparison of sEPSC and mEPSC mean amplitudes in layer III.*

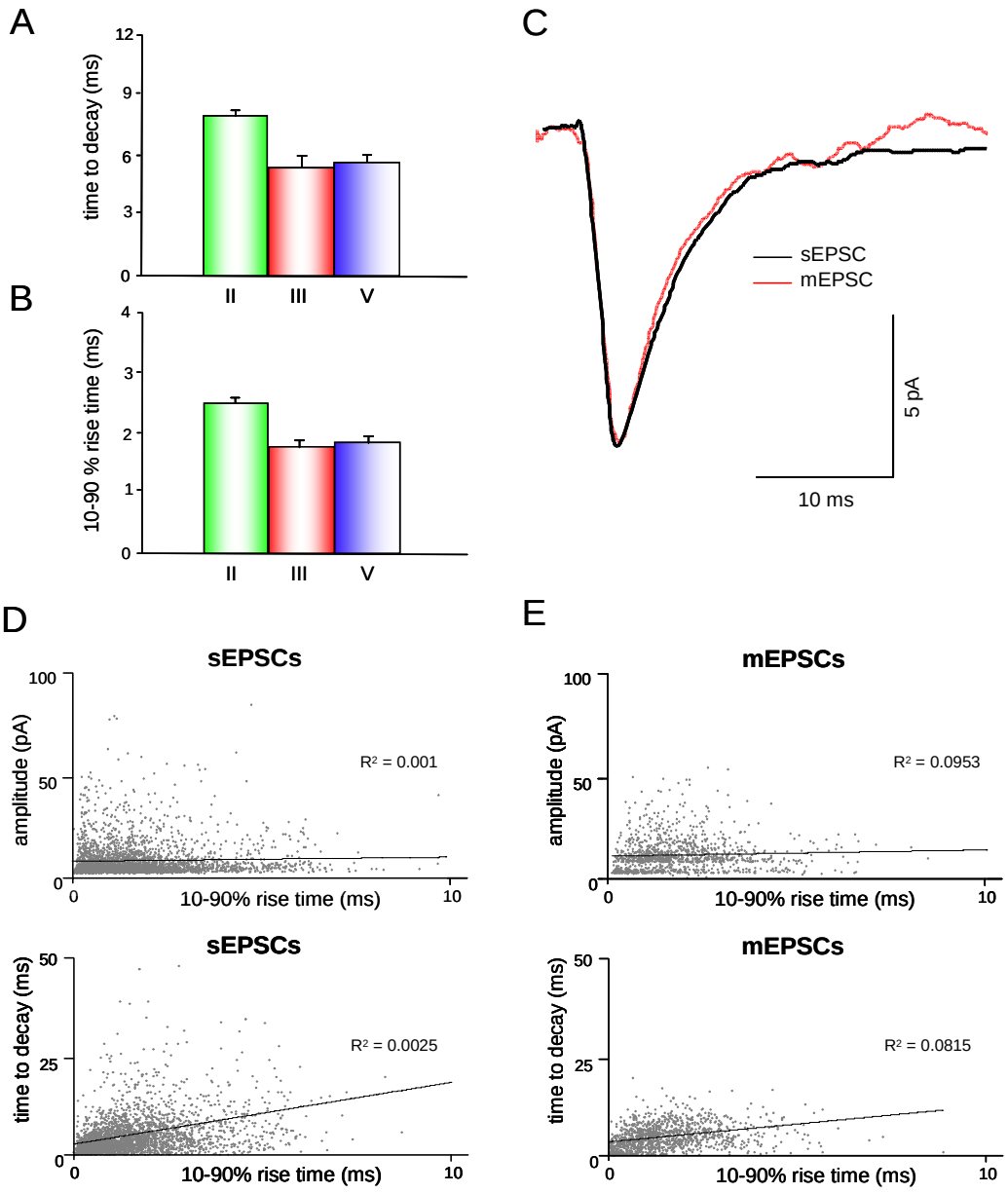


Figure 3.4. Kinetics of sEPSCs and mEPSCs in EC neurones. *A, B: The graphs shows mean decay time (A) and 10-90% rise time (B) of layer III sEPSCs, compared to layer II and V (data from Berretta and Jones, 1996a). C: Trace of average EPSC (n=60; scaled for amplitude) in the presence (red) and absence (black) of TTX (1 µM). D, E: Graphs showing correlations between rise time and decay and also between rise time and amplitude for sEPSCs (D) and mEPSCs (E).*

3.3.1.5. Pharmacology

Bath perfusion with NBQX (10 µM) completely abolished sEPSCs in layer III neurones (Figure 3.6) indicating that they are mediated primarily by non-NMDAr, most probably AMPAr. In confirmation of this, SYM 2206 (20 µM) or GYKI 53665 (25 µM) also abolished the sEPSCs (not shown). In contrast, neither UBP 302 (20 µM) nor 2-AP5 (50 µM) had any effect, indicating that GluR5 KAr or NMDAr do

not contribute markedly to sEPSCs (further analysis included in Sections 4.3.1.2 and 4.3.6.). However, unlike in layers II and V (Beretta and Jones, 1996a), there was no real evidence of slower, NMDA receptor mediated sEPSCs in the presence of NBQX at -60mV. However, when the holding potential was shifted towards positive values slow time course events (at +20 mV DT - 18.7 ± 3.2 and RT – 2.9 ± 0.6) did become apparent. These events reversed at around 0 mV and increased in amplitude at positive holding potentials. These are characteristics of NMDA receptor mediated events. In confirmation of this, they were abolished in the presence of 2-AP5 (50 µM; Figure 3.6C).

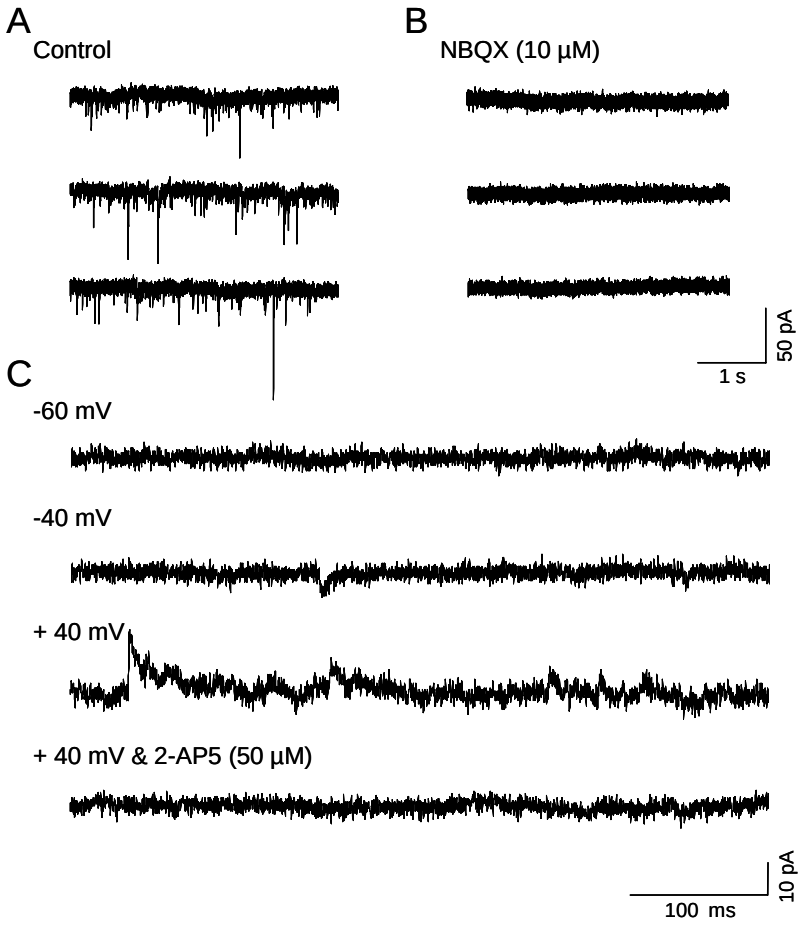


Figure 3.5. Pharmacology of sEPSCs in layer III neurones. *The traces are voltage clamp recordings from 3 typical layer III neurones in control (A) and after addition of NBQX (B). NBQX (10 µM) effectively abolished sEPSCs. C: Consecutive traces from a typical layer III neurone in the presence of NBQX at -60, -40 and +40 mV and after the addition of 2-AP5 (50 µM).*

3.3.2. Characterisation of sIPSCs

Woodhall *et al*. (2005) presented a detailed comparison of the properties of sIPSCs in layer II and layer V of the medial EC. I have now examined sIPSC properties in layer

III recorded under practically identical conditions. In the recording conditions employed (symmetrical Cl^-) sIPSCs are manifest as fast inward currents at a holding potential of -60 mV (Figure 3.6A). These events reverse at 0 mV, to become outward currents at positive potentials. Again, recordings have been made in many neurones, but a sample of high quality neurones have been selected for use in this analysis.

3.3.2.1. Amplitude and frequency of sIPSCs

The detailed analysis of sIPSC characteristics were conducted on representative whole cell recordings made from a total of 19 neurones in layer III of the medial EC. The mean IEI of sIPSCs in the 19 neurones was 112 ± 21 ms which corresponds to a mean sIPSC frequency of 15.5 ± 2.8 Hz (Figure 3.6). This is of the same order that was previously reported for layer II cells (IEI – 87 ± 2 ms; Woodhall *et al.*, 2005), but substantially faster than in layer V (IEI – 404 ± 10 ms; Woodhall *et al.*, 2005).

sIPSCs in layer II and layer V show an amplitude distribution represented by histograms with a single peak, skewed toward larger amplitude events (Woodhall *et al.*, 2005). The same was seen in layer III (figure 3.6C). The mean amplitude of sIPSCs in the sample of 19 neurones in layer III was 30.9 ± 2.0 pA. This is larger than that reported for both layer II and layer V (24.0 ± 1.8 pA and 25.7 ± 3.9 pA, respectively; Figure 3.6D).

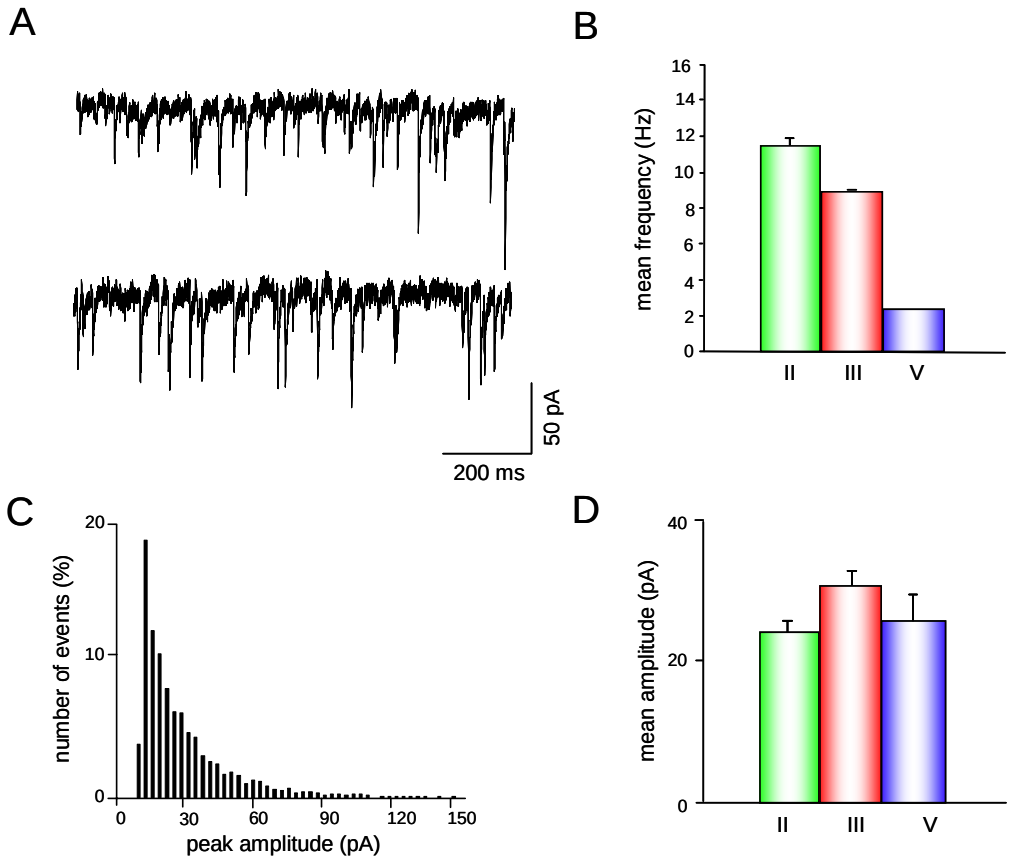


Figure 3.6. Comparison of sIPSC amplitude and frequency in EC neurones. *A: The traces are consecutive voltage clamp recordings from a typical layer III neurone. The downward deflections are sIPSCs. B: The graphs show the mean frequency of sIPSCs in layer III, compared to values for layers II and V (data from Woodhall et al., 2005). C: Frequency distribution of sIPSC amplitudes in layer III neurones (bin width = 3). The distribution shows a single peak, skewed toward larger amplitude events. D: Comparison of average sIPSC amplitudes in layer III with layer II and V (data from Woodhall et al., 2005).*

3.3.2.2. IPSC bursts

As with sEPSCs, sIPSCs can occur in bursts in EC neurones. In the previous analysis of sIPSCs in layers II and V a major difference was found to be in the frequency of bursts (defined as at least 3 events in a 50 ms time frame). These were found to occur at a much higher frequency in layer II than in layer V (inter-burst interval - 653 ± 79 ms vs. 5447 ± 627 ms; Woodhall *et al.*, 2005). I have examined the characteristics of sIPSC bursts in layer III neurones (Figure 3.7A). In the 19 layer III neurones, I found the inter-burst interval to be 574 ± 48 ms, a very similar frequency to that seen in layer II and much more frequent than V (Figure 3.7B). However, the number of events per burst was closer to that seen in layer V (3.5 ± 0.1 compared to 5.8 ± 0.7 in layer II and 3.7 ± 0.2 ms in layer V; Figure 3.7C). Further analysis of the bursts

revealed that the frequency of IPSCs within bursts was slightly faster in layer III than that seen in either layers II or V (15 ± 0 ms compared to 19 ± 1 ms in layer II and 19 ± 1 ms in layer V) but that the mean duration of the bursts was less (37 ± 1 ms compared to 93 ± 9 ms in layer II and 80 ± 5 ms in layer V).

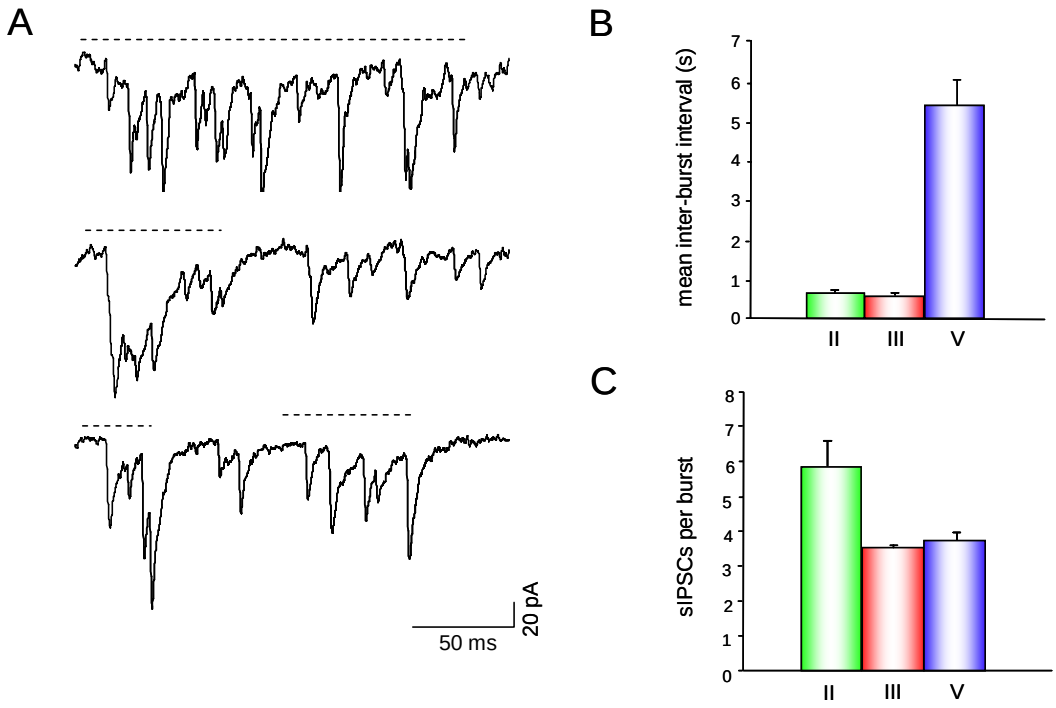


Figure 3.7. Bursts of sIPSCs in layer III neurones: Comparison to layer II and V. *A: The traces are voltage clamp recordings showing bursts of sIPSCs (defined as at least three events within a 50 ms time frame) from typical layer III neurones. Bursts are indicated by the dotted line above the trace. B,C: Histograms showing mean inter-burst interval (B) and number of sIPSCs per burst (C) for layer III, compared to layer II and V (data from Woodhall et al., 2005).*

3.3.2.3. Effects of TTX on sIPSCs.

As with glutamate release, GABA release consists of spontaneous events (action potential driven) and miniatures (action potential-independent). The IEI of sIPSCs in the presence of TTX (1 μM) approximately doubled from 112 ± 21 ms to 238 ± 44 ms (n=10; KS test – $P<0.0001$; Figure 3.8). This represents a decrease in frequency from 15.5 ± 2.8 Hz to 6.0 ± 1.1 Hz, indicating that approximately half of the IPSCs in layer III are action potential-dependent. This effect is similar to that seen in layer V where the IEI more than doubled (from 368.2 ± 13.1 ms to 979.9 ± 31.5 ms; Woodhall *et al.*, 2005). In contrast, in layer II, TTX had little or no effect (Woodhall *et al.*, 2005) showing that action potential driven release is almost non-existent in layer II in the slice preparation. In terms of absolute numbers of sIPSCs, layer III neurones showed

a much greater decrease than layer V in the presence of TTX, as a result of their higher sIPSC frequency to begin with (Figure 3.8C).

Neither layer II nor V showed any significant difference in amplitude comparing sIPSCs with mIPSCs (Woodhall *et al.*, 2005). In contrast, in layer III, application of TTX also caused a shift in the cumulative amplitude distribution (KS test – $P<0.0001$; Figure 3.8D) towards smaller events, suggesting a loss of large amplitude events. This was also reflected by the decrease in mean amplitude of IPSCs in the presence of TTX (from 30.9 ± 2.1 pA to 23.7 ± 1.7 pA; n=10; t test – $P<0.01$; Figure 3.8E).

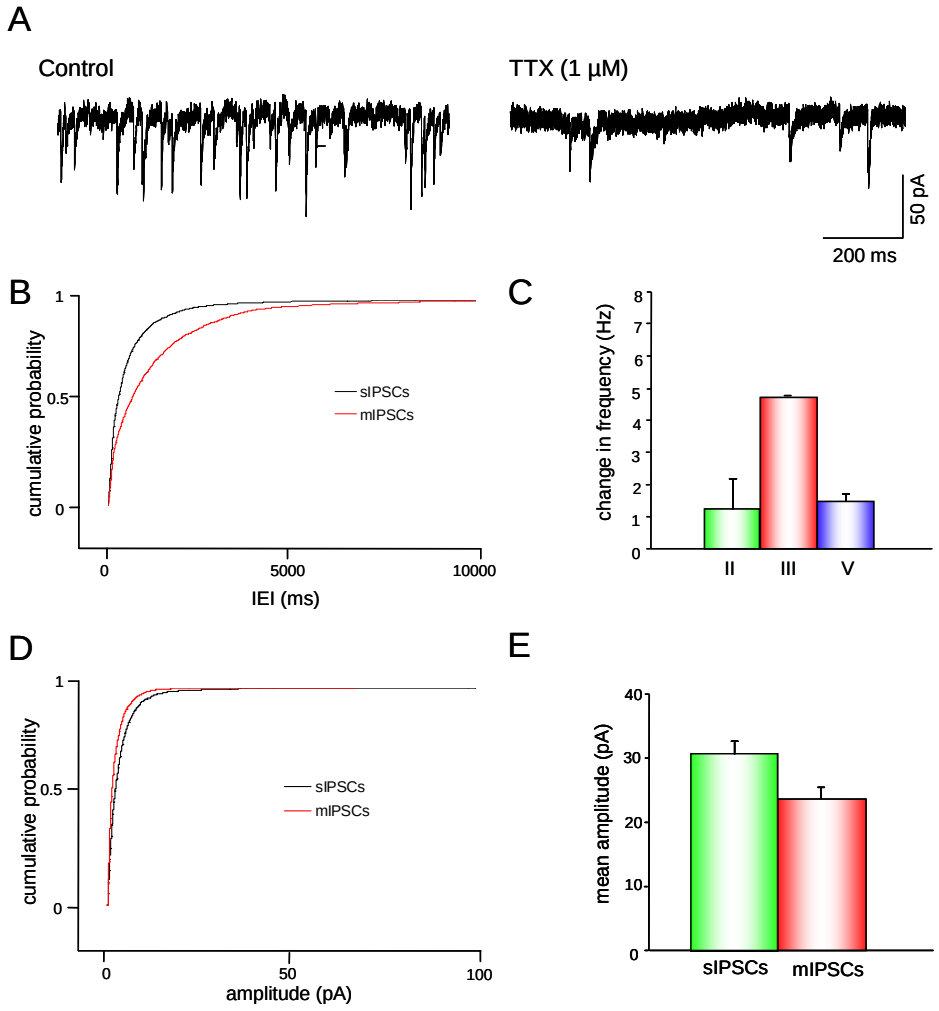


***Figure 3.8*. Comparison of sIPSCs and mIPSCs in EC neurones.** *A: The traces are consecutive voltage clamp recordings from a typical layer III neurone in the absence and presence of TTX (1 µM). B: Cumulative probability graph of IEI (n=10) of sIPSCs and mIPSCs in layer III neurones. C: Histogram showing the change in frequency after addition of TTX (frequency of sIPSC-frequency of mIPSC) in layer III neurones, compared to layer II and V neurones (data from Berretta and Jones, 1996a). D: Cumulative probability graph of amplitude (n=10), comparing mIPSC and sEPSC in layer III. E: Comparison of sEPSC and mIPSC mean amplitudes in layer III.*

3.3.2.4. Kinetics of sIPSCs

sIPSCs in layer III had a mean decay time of 15.8 ± 1.5 ms, which is longer than those seen in the other layers (layer II – 10.5 ± 0.1 ms vs. 10.6 ± 0.11 in layer V; Figure 3.8A). Mean 10-90% rise times (2.2 ± 0.2 ms) were broadly similar to those reported for both layer II and layer V (2.0 ± 0.03 ms and 1.9 ± 0.03 ms respectively; Woodhall *et al.*, 2005; Figure 3.9B). Application of TTX, appeared to slightly alter time to decay time (from 5.9 ± 0.5 to 4.9 ± 0.3) and 10-90% rise time (from 2.2 ± 0.5 to 2.4 ± 0.2) but the differences were not significant.

As has been suggested for sEPSCs, correlation of kinetic parameters of postsynaptic inhibitory events may enable the identification of inputs to different cell surface domains (Maccaferri *et al.,* 2000). I therefore looked for correlations between rise time and decay, and also between rise time and amplitude for the pooled data of both sIPSCs and mISPCs. Such analysis in layer II and layer V neurones failed to identify any significant correlations between rise time and amplitude, or rise and decay time in either population (Woodhall *et al*., 2005). Figure 3.9D and E clearly shows that there were also no significant correlations for these parameters in layer III either, with r^2 values less than 0.15. Again this is unsurprising due to the inclusion of Cs in the patch solution. Although Woodhall *et al*., (2005) could separate populations of neurones in both layer II and V on the basis of decay time, this analysis has not been attempted here due to the relatively small number of layer III cells available.

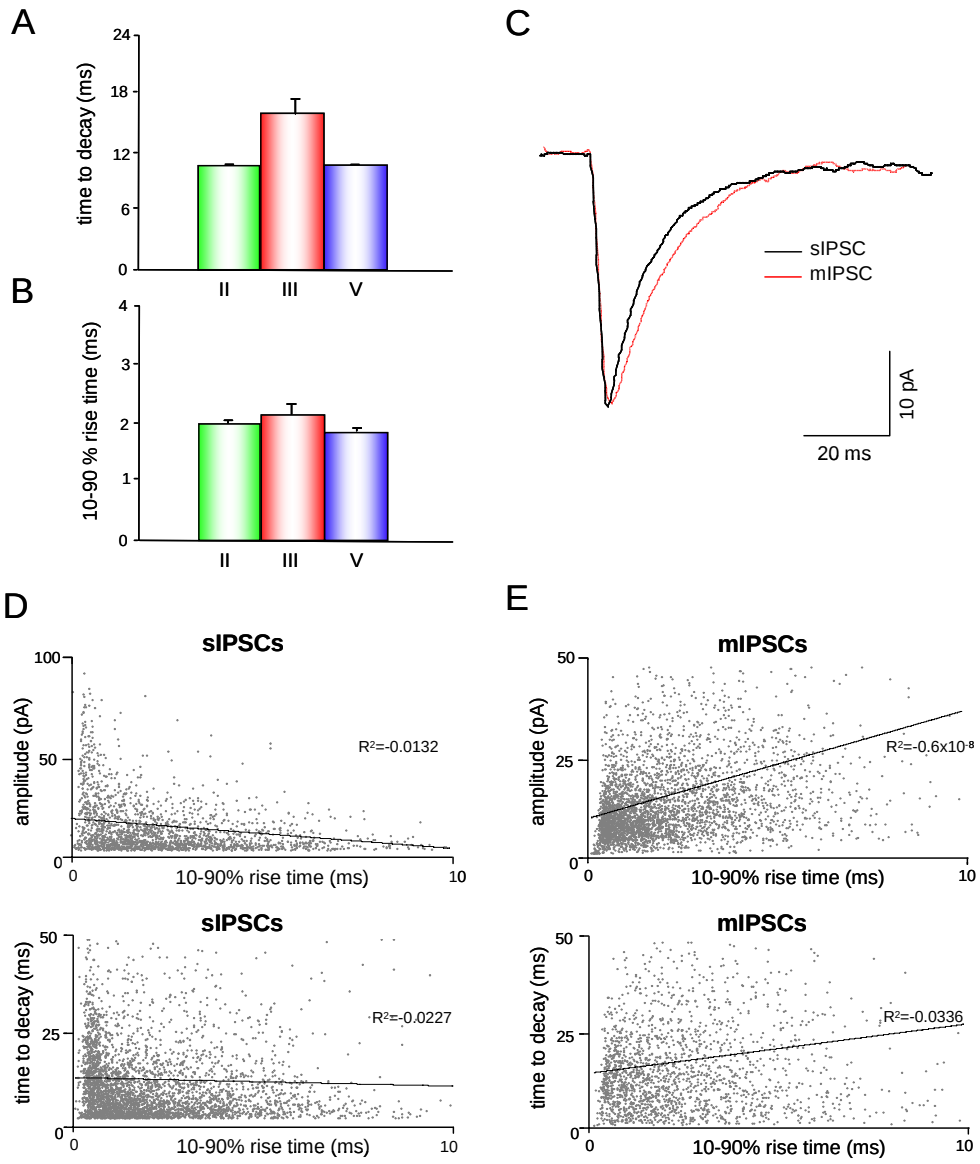


Figure 3.9. Kinetics of sIPSCs and mIPSCs in EC neurones. *A, B: The graphs shows mean decay time (A) and 10-90% rise time (B) of layer III sIPSCs, compared to layer II and V (data from Woodhall et al., 2005). C: Trace of average EPSC (n=60) in the presence (red) and absence (black) of TTX (1 µM). D, E: Graphs showing correlations between rise time and decay and also between rise time and amplitude for sIPSCs (D) and mIPSCs (E).*

3.3.2.5. Pharmacology

Bath application of gabazine (20 µM) rapidly abolished sIPSCs (Figure 3.10A,B) indicating that they are primarily mediated by GABA acting at $GABA_A$r. It should be noted that addition of gabazine had no effect on the holding current. This indicates that the $GABA_A$ receptor mediated standing tonic current in a variety of other neurones (Brickley *et al.*, 1996; Nusser and Mody, 2002) is not found in layer III.

These results are similar to those in layers II and III where gabazine abolished sIPSCs without affecting holding current (Woodhall *et al.*, 2005).

Bath application of strychnine (1 µM) appeared to slighty increase the IEI of sIPSCs in layer III neurones from 168 ± 117 ms to 198 ± 134 ms. This effect was marginal, but in the small sample of neurones tested (n=3) just reached significance when assessed by KS analysis of cumulative probability ($P<0.01$; Figure 3.10C). Thus, there may be a small contribution of glycine mediated sIPSCs in layer III (in contrast to layers II and V; Woodhall *et al.*, 2005). Previous work from this laboratory has suggested that presynaptic NMDAr may facilitate GABA release in layer II but not layer V. However, there are not tonically active as 2-AP5 has no effect on sIPSC frequency (Jones and Woodhall, 2005). Similar results were found in layer III neurones where 2-AP5 (50 µM) had no effect on sIPSC frequency (Figure 3.10D).

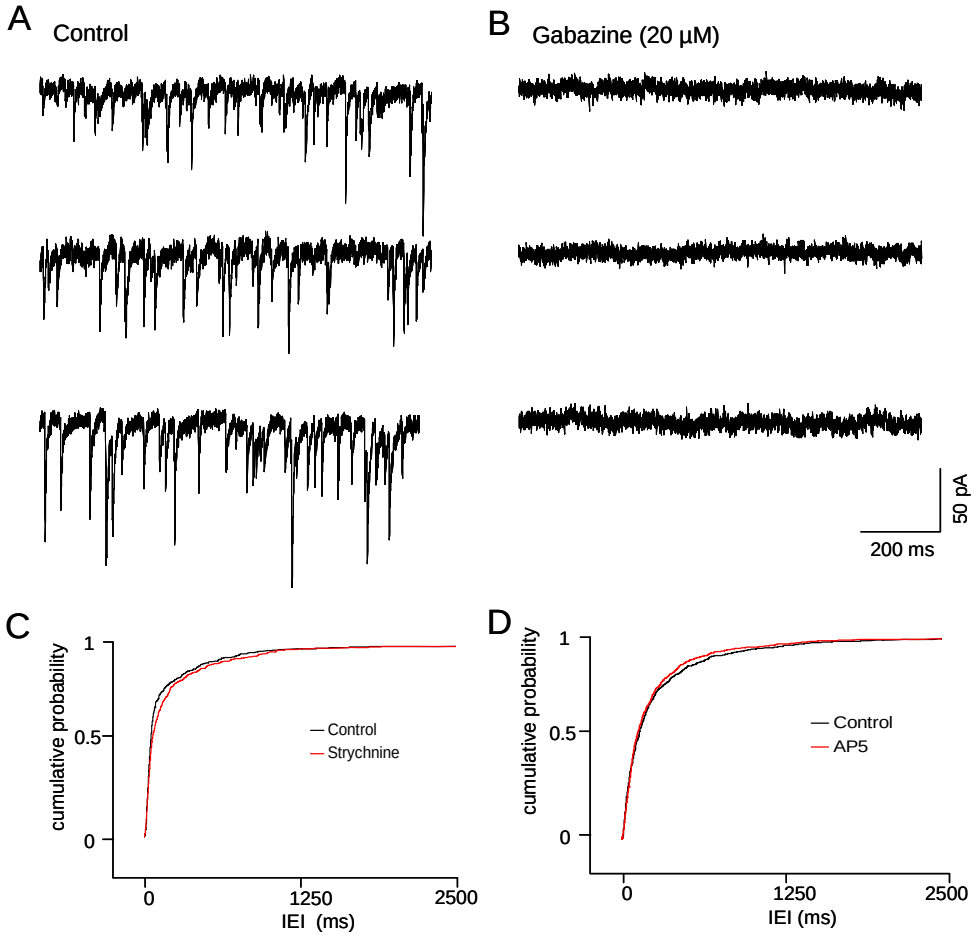


Figure 3.10. Pharmacology of sIPSCs in layer III neurones. *The traces are voltage clamp recordings from 3 typical layer III neurones in control (A) and after addition of gabazine (B). Gabazine (20 µM) effectively abolished sIPSCs. C: Cumulative probability graph of IEI (n=3) comparing control to strychnine conditions. D: Cumulative probability graph of IEI (n=4) comparing control to 2-AP5 conditions.*

3.4. Discussion

Examination of spontaneous synaptic activity in layer III revealed some interesting differences from the deeper and more superficial layers. This thesis has focused on layer III due to its involvement in TLE (e.g. Du and Schwarcz, 1992; Du *et al*., 1995; Tolner *et al*., 2005) and SWO (Cunningham *et al*., 2006). In view of the relative lack of physiological and pharmacological studies carried out in layer III in comparison to the other layers of the EC, it is interesting to consider how the properties of spontaneous activity may affect synaptic behaviour in relation to generation of synchronous behaviour.

3.4.1. sEPSCs

Neurones in layer III exhibited continuous spontaneous excitatory activity and the characteristics of sEPSCs were generally similar to those recorded in the other layers (Berretta and Jones, 1996a; Woodhall *et al*., 2001a; Jones and Woodhall, 2005) in that they were abolished by NBQX (and other AMPAr antagonists) and therefore are largely mediated by non-NMDAr, primarily AMPAr. However, more in depth analysis indicated that there are obvious differences between the spontaneous activity in layer III compared that in other layers of the EC. What is perhaps surprising is that there are substantial differences between layer III and layer II, which provide the afferent input to the hippocampus, and substantial similarities to layer V, which is the major relay of hippocampal output (Dugladze *et al*., 2001; Insausti *et al*., 1997; Witter *et al*., 1989a, b; Sorensen, 1985).

The most pronounced difference between the sEPSCs in layer III compared to the other layers is the much higher frequency of events. It is also noteworthy that bursts of activity occurred frequently in layer III. Thus, the higher levels of spontaneous glutamate release in layer III may be due to a greater number of synaptic contacts onto these neurones. However, it seems more likely to be associated with an increased firing rate and/or connectivity of the neurones in layer III, since TTX had a pronounced effect (decreasing frequency five fold). Paired recordings of single axon connections have shown a high degree of connectivity between neurones in layer III, somewhat higher than layer V, and considerably higher than layer II (Dhillon and Jones, 2001). Thus, it could be postulated that the high frequency of sEPSCs in layer

III is a reflection of high baseline firing rates in principal neurones connected by extensive recurrent excitatory collaterals. There is also evidence for a high degree of electrical coupling between layer III principal neurones via gap junctions (Dhillon and Jones, 2001), which could further contribute to the high level of baseline glutamate release.

The distribution of sEPSC amplitudes in layer III showed a single peak, skewed toward larger amplitudes, similar to that seen in layers II and V (Berretta and Jones, 1996a). Also, similar to the other layers, it is unlikely that this variability is due to sEPSCs originating from different sites on the dendritic tree, as amplitudes showed no correlation with sEPSC kinetics, as would have been expected if synaptic location was a major factor (McBain and Dingledine, 1992). Therefore, it is more likely to be caused by variability at individual synapses. Although differences between layers were small, average peak amplitude, was similar to that in layer V neurones and larger than layer II (Berretta and Jones, 1996a). Berretta and Jones (1996a) suggested that the differences in amplitude of sEPSCs between layers II and V was most likely due to a greater activity-dependent presynaptic release in the latter. Activity-dependent sEPSCs in layer V are likely to be mediated by multi-quantal release of glutamate. In layer II there was no change in the amplitude distribution in TTX and therefore both the action potential-independent and dependent release are probably mediated by the release of single quanta. TTX does not completely abolish large amplitude events in layer V (Berretta and Jones, 1996a) and the same is true in layer III. These data suggest something else may be contributing to the difference in EPSC amplitude between layers in the EC. As there was no correlation between amplitude and rise time it seems unlikely that differences in electrotonic length are responsible for difference in amplitude of sEPSCs between layers. As noted above, recurrent excitatory connectivity is high in layers III and V but low in II (Dhillon and Jones, 2001). It may be that the large amplitude, activity-dependent events reflecting release from other excitatory afferents.

The kinetics of sEPSCs in layer III are very similar to layer V, rather than the much slower kinetics of layer II sEPSCs. Berretta and Jones (1996a) suggested that electrotonic filtering was a factor in the different kinetics of layers II and V, as they estimated superficial neurones have greater electrotonic length. The majority of principal neurones in layer III are spiny stellates (Alonso and Klink, 1993, Jones,

1993), which have relatively complex dendritic arborisations compared to the principal neurones in layer V, which are medium-size pyramidal neurones (Buckmaster *et al*., 2004; Hamam *et al*., 2002). Layer III neurones are also medium sized pyramidal neurones (Gloveli *et al*., 1997; Dhillon and Jones, 2001; Buckmaster *et al*., 2004; Tahvidori and Alonso, 2005). The similarity in morphology between layer III and V neurones probably means they are similar electronically, as has been estimated previously (Dhillon and Jones, 2001), and may explain similar kinetics of sEPSCs in the two layers.

There may also be a difference in the contribution of NMDAr in layer III compared to layers II and V. When AMPAr mediated sEPSCs were blocked in layers II and V, residual pure NMDAr mediated sEPSCs occured even at negative holding potentials (Berretta and Jones, 1996a). The authors suggested that this indicated a strong NMDAr mediated excitation in these neurones (greater in layer V than layer II) compared to other brain regions where NMDAr mediated sEPSCs could only be detected at positive holding potentials (e.g. CA3 – MacBain and Dingledine, 1992). In layer III observation of NMDAr mediated sEPSCs required somewhat more positive holding potentials. The difference was small, and its significance unclear. One possibility is that there may be differences in subunit composition of postsynaptic NMDAr, and hence voltage sensitivity (see Cull-Candy *et al*., 2001), in layer III compared to II and V. It is also interesting that 2-AP5 reduces frequency in both layer II and V (Beretta and Jones, 1996a; Woodhall *et al*., 2001a) but did not in layer III. This may indicate that tonic facilitation of glutamate release by presynaptic NMDAr (Beretta and Jones, 1996b; Woodhall *et al*., 2001a) is not operative in layer III (see also Section 4.3.6.).

The GluR5 KAr antagonist UBP 302, failed to alter sEPSC frequency, amplitude or kinetics of sEPSCs in layer III (or layer V, see Section 7.3.3.). Thus, it is clear that these receptors (either pre- or postsynaptically) are unlikely to be accessed by spontaneously released glutamate. In any case, sEPSCs were abolished by NBQX or GYKI 53665 indicating that KAr of any subunit composition were unlikely to contribute to spontaneous excitation. This contrasts with studies in CA3, where GYKI-resistant mEPSCs have been observed, which are presumed to result from KAr activation (Cossart *et al*., 2002).

3.4.2. sIPSCs

Neurones in layer III also exhibited continuous spontaneous inhibitory activity. The characteristics of these sIPSCs were generally similar to those recorded in the other layers of the EC (Woodhall *et al.,* 2005). They were completely abolished by gabazine, and therefore are mediated by $GABA_A$r. Also, as in layer II and V, gabazine had no effect on holding current indicating an absence of the tonic background current that is a feature of GABAergic synapses in other brain regions (Brickley *et al.,* 1996a; Nusser and Mody, 2002). It should be noted that strychnine did slightly reduce the frequency of sIPSCs in layer III. This could indicate that a few sIPSCs may be glycine receptor mediated. However, the difference was very small and it should be stressed that gabazine essentially abolished sIPSCs so any contribution of glycine receptors is likely to be insignificant in terms of total background inhibition.

Interestingly, sIPSCs in layer III had clearly larger amplitudes and longer decay and rise times than the events previously described for the other layers of the EC. In the presence of TTX the amplitude of sIPSCs was reduced and was no longer different from the average amplitude seen in layers II and V. However, time to decay and rise time was still significantly larger. The change in amplitude in the presence of TTX, was not seen in layers II and V (Woodhall *et al*., 2005). It may be that activity-dependent sIPSCs in layer III are the result of release of multiple quanta whereas in layers II and V they are mediated by the release of single quanta. This does not, however, explain the slower kinetics of sIPSCs in layer III as these are unaffected by the addition of TTX. However, it has been shown in several brain areas, including the hippocampus (Pearce, 1993) that $GABA_A$r can mediate much slower IPSCs (decay time 30-70ms) as well as the more typical fast IPSCs (3-8 ms) which may represent a different subunit composition of postsynaptic recptors. In layer III neurones, approximately 25% of the IPSCs recorded have decay times greater than 20 ms and therefore the longer average decay time in these neurones when compared to layers II and V may indicate a greater contribution of these slower $GABA_A$ mediated IPSCs. There has also been some recent evidence to suggest that the slower $GABA_A$ mediated IPSCs may result from the activation of specific types of interneurone (Szabadics *et al.,* 2007) and it is possible that the difference in rise and decay times in the EC between layers may be due to the differential contribution from interneurone subtypes.

Woodhall *et al*. (2005) noted that the most pronounced difference between layers II and V was in frequency of sIPSCs, with layer II sIPSCs occurring four times faster than those in layer V. They suggested that it was likely that this was due to a greater number of interneurones and/or more synaptic contacts per interneurone in layer II. sIPSC frequency in layer III was if the same order as in layer II. My data would indicate that the same applies to layer III, with pyramidal cells receiving a large number of GABAergic inputs, similar to layer II and much more pronounced than layer V. This would fit with the general density of GABAergic interneurones revealed by immunohistochemical studies (Kohler and Chan-Palay, 1982, 1983; Lotstra and Vanderhaeghen, 1987; Rogers, 1992; Wouterlood *et al*., 1995; Fujimaru and Kosaka, 1996; Miettinen *et al*., 1996, 1997; Mikkonen *et al*., 1997; Wouterlood and Pothuizen, 2000; Wouterlood *et al*., 2000; Arellano *et al*., 2002).

One of the major differences between layers was in the contribution of activity-dependent vs. independent release. Thus, in layer II, TTX had little effect on sIPSC frequency whereas it reduced it by around 60% in layer V (Woodhall *et al*., 2005). In layer III, the very high initial frequency of sIPSCs was halved by TTX. Thus, layer V neurones undergo a low level of spontaneous inhibition which is primarily driven by interneurone firing. Layer II neurones have a very high level of background inhibition, that is largely independent of spontaneous firing in interneurones. Whereas, layer III has a high level of background inhibition but a large proportion of this results from spontaneous interneurone firing. It is apparent that oscillatory activity in networks can depend on interneurone-interneurone and/or principal-interneurone interactions (Cunningham *et al*., 2003). The high level of action potential driven inhibition and excitation in layer III may indicate a high level of interconnectivity in the network of this layer. The pronounced burst activity in both excitatory and inhibitory activity may also reflect this interconnectivity. This may be associated with the susceptibility of layer III to synchrony and oscillations (Cunningham *et al*., 2003; 2006a; b).

CHAPTER 4

LOCALIZATION AND FUNCTION OF KAR AT GLUTAMATERGIC SYNAPSES IN LAYER III

4.1. Introduction

In the previous Chapter I described the properties of spontaneous excitatory events in layer III neurones. Pharmacogical data suggested that KAr were unlikely to contribute directly to mediating events occurring in response to spontaneous glutamate release. However, because KAr have been shown to be widely involved in excitatory transmission elsewhere, the aim of the work in this Chapter was to identify the presence and role of presynaptic KAr at glutamatergic synapses in layer III of the EC. A major role of KAr is to presynaptically control neurotransmitter release at excitatory synapses throughout the CNS (Kidd *et al*., 2002; Schmitz *et al*., 2001; Lauri *et al*., 2001; Jin *et al*., 2006; Lauri *et al*., 2006; Campbell *et al*., 2007). The nature of this control has been the subject of controversy, with evidence for both facilitation (Schmitz *et al*., 2001) and inhibition (Jin *et al*., 2006) of glutamate release.

The area where presynaptic KAr have been most intensely studied is in the hippocampus (see review by Kullmann, 2001). The first evidence for the presence of presynaptic KAr came from studies investigating the effects of KA on synaptosomes isolated from the hippocampus. Malva *et al*. (1995) studied the effects of KA on intracellular free Ca^{2+} concentration in hippocampal synaptosomes and concluded that there were presynaptic KAr modulating Ca^{2+} levels in nerve terminals in the CA3 region, as stimulation with KA caused an increase in intracellular Ca^{2+}. Chittajallu *et al*. (1996) showed that, in hippocampal synaptosomes, brief exposure to KA inhibited Ca^{2+}-dependent [^{3}H]L-glutamate release, an effect that was reversed by KAr antagonists but not selective AMPAr antagonists. This provided the first evidence that presynaptic KAr could inhibit glutamate release. These authors took this further by showing that micromolar concentrations of KA, in the presence of an AMPAr selective antagonist, reduced NMDAr mediated EPSCs in CA1 neurones. Other evidence also suggests that presynaptic KAr act to depress glutamate release in other brain regions including the nucleus accumbens (Casassus and Mulle, 2002), in the cortex during early postnatal development (Kidd *et al*., 2002), and at primary sensory afferent synapses in the dorsal horn of the spinal cord (Kerchner *et al*., 2001).

In contrast, presynaptic KAr have also been shown to facilitate glutamate release. This was demonstrated in both hippocampal (Malva *et al*., 1998) and cortical synaptosomes (Perkinton and Sihra, 1999). In agreement with Malva *et al*. (1995;

1998), Schmitz *et al*. (2001) provided direct evidence for a presynaptic KAr in CA3, which act to potentiate glutamate release. They suggested that it was KAr activation which was responsible for the frequency facilitation at this synapse, which was previously thought to be due to residual Ca^{2+}. Subsequently, KAr have been found to facilitate glutamate release at many excitatory synapses throughout the CNS, including in the amygdala (Li *et al*., 2001), the cerebellum (Delaney and Jahr, 2002) and neocortex (Campbell *et al*., 2007).

Interestingly, at many synapses where presynaptic KAr facilitates glutamate release they have also been found to have an inhibitory action at higher concentrations of agonist (Schmitz *et al*., 2001; Delaney and Jahr, 2002; Campbell *et al*., 2007). This bidirectional modulation of neurotransmitter release was first described at inhibitory synapses (Rodriguez-Moreno *et al*., 1997), but has now been shown to occur at excitatory synapses, both when high concentrations of exogenous agonists were applied, and also when endogenous glutamate levels are increased by higher frequency stimulation (Schmitz *et al*., 2001; Delaney and Jahr, 2002). However, not all glutamatergic synapses where KA autoreceptors facilitate transmitter release exhibit this bidirectionality. In some synapses increasing concentrations of agonist continue to potentiate release (Li *et al*., 2001).

The subunit composition of presynaptic KAr located on glutamatergic terminals in different brain regions remains a source of much debate. At mossy fibre synapses alone GluR6 and 7 and KA1 and 2 have all been suggested to be present (Breustedt and Schmitz, 2004; Pinheiro *et al*., 2007; Darstein *et al*., 2003 respectively), whereas the GluR5 subunit is thought to be involved at excitatory synapses on layer II/III pyramidal neurones in the prefrontal cortex (Campbell *et al*., 2007) and cingulate cortex (Wei *et al*., 2005).

KAr have also been found to contribute to the postsynaptic current at glutamatergic synapses in many areas of CNS, including the hippocampus (Castillo *et al*., 1997; Vignes and Collingridge, 1997), somatosensory cortex (Kidd and Isaac 2006), prefrontal cortex (Campbell *et al*., 2007), cingulate cortex (Wei *et al*., 2005) and perirhinal cortex (Park *et al*., 2006). Again, postsynaptic KAr mediated response were initially identified in the mossy fibre pathway, on CA3 pyramidal neurones (Castillo *et al*., 1997; Vignes and Collingridge, 1997). Synaptic responses mediated by KAr

share two main features, the synaptic current is smaller than that mediated by AMPAr (about 10% of the total peak current) and it has significantly slower decay kinetics (Castillo *et al*., 1997; Vignes *et al*., 1997; Frerking *et al*., 1998). In addition, KAr desensitize completely and rapidly in response to glutamate, with a time constant of approximately 5 ms (Dingledine *et al*., 1999).

Postsynaptic KAr on CA3 neurones were initially thought to contain GluR5 since the eEPSC could be reduced by a number of GluR5 antagonists (Vignes *et al*., 1997). In addition, a contribution of GluR6 subunits was indicated as the EPSC is no longer present in GluR6 knockout mice (Mulle *et al*., 1998). However, there has been debate as to whether the postsynaptic receptors do contain the GluR5 subunit at this synapse as this does not fit with the mRNA expression pattern (Paternain *et al*., 2000) and the EPSC is still present in GluR5 knockout mice (Contractor *et al*., 2000). Contractor *et al*. (2003) have also suggested a role for the KA2 subunit at mossy fibre synapses, because in KA2 subunit deficient mice, although the KAr EPSC is still present, it has a significantly decreased decay time, indicating a change in affinity of KA for the receptor.

Since their discovery in the mossy fibre pathway, postsynaptic KAr have also been identified at other synapses, including in the somatosensory cortex (Kidd and Isaac, 2001). The KAr mediated EPSC at these synapses exhibited the slow decay kinetics seen in the mossy fibre synapse (Castillo *et al*., 1997). Kidd and Isaac (2001) determined that the slow decay is an intrinsic property of KAr and not due to an extrasynaptic location as previously suggested (Lerma, 1997), as neither inhibition nor facilitation of glutamate clearance has any effect on kinetics. KAr mediated synaptic responses have also been found in the superficial layers perirhinal cortex (Park *et al*., 2006), where they are regulated by tonically activated mGluR5. In addition, postsynaptic KAr have been found on hippocampal interneurones, where they are activated by single stimuli, possibly indicating that the receptors are not extrasynaptic at these synapses (Cossart *et al*., 1998).

More recently, a KAr-mediated EPSC has been demonstrated in the superficial layers of the EC (West *et al*., 2007). It appears to be more pronounced in layer III neurones compared to layer II. The KAr eEPSC in the EC displays the same small amplitude and slow kinetics as noted in other regions (Castillo *et al*., 1997; Vignes *et al*., 1997;

Frerking *et al.*, 1998). However, no information concerning the possible subunit composition of these receptors is currently available.

Thus, the aims of this chapter were: 1) to determine if presynaptic KA autoreceptors are present in layer III of the EC; 2) if KAr autoreceptors are present what is their function; 3) to elucidate the possible subunit composition of presynaptic KAr in layer III; 4) to determine whether postsynaptic KAr contribute to the evoked EPSC; 5) to elucidate the possible subunit composition of the postsynaptic receptors.

4.2. Methods

General recording methods were as described in Chapter 2. To investigate the function of KAr specific use of the GluR5 selective antagonist, UBP 302, and the GluR5 selective agonist, ATPA, were made.

Isolation of the postsynaptic KAr mediated synaptic current was achieved by using a cocktail of 2-AP5 (50 µM), SR 95531 (20µM), CPG 55845A (5µM) and either SYM 2206 (20 µM) or GYKI 53655 (25µM) to block NMDAr, $GABA_Ar$, $GABA_Br$ and AMPAr mediated currents. A stimulus train of either 50 or 100 Hz was used to evoke an EPSC large enough for analysis. The mean amplitude and time to decay were calculated for three consecutive KAr eEPSCs.

4.3. Results

4.3.1. Presynaptic effects

4.3.1.1. Effects of ATPA on sEPSCs

In the first set of experiments I determined the effect of activating GluR5 KAr with the specific agonist, ATPA (0.1 µM and 0.5 µM). In the presence of 0.1 µM ATPA, there was a decrease in IEI from 174 ± 32 ms to 78 ± 7 ms (KS test - $P<0.001$, n=7) reflecting a change in frequency from 5.8 ± 1.2 Hz to 9.9 ± 1.0 Hz. A further decrease to 60 ± 9 ms occurred (14.6 ± 2.8 Hz) after addition of 0.5 µM ATPA (KS test - $P<0.001$, n=5; Figure 4.1A). Thus, the KAr agonist induced a concentration-dependent increase in frequency of sEPSCs, with the higher concentration eliciting a

3-fold rise. There was no change in the amplitude or kinetics. Averaged sEPSCs for control and in the presence of ATPA (n=60) can be seen in Figure 4.1B and table 4.1 summarises IEI, amplitudes and kinetics of sEPSCs in control and in the presence of ATPA. The increase in frequency of sEPSCs with no effect on amplitude or kinetics may be an indication that the agonist was acting presynaptically to enhance glutamate release.

To determine whether the effect of ATPA was on presynaptic GluR5 KAr on the glutamate terminals or was driven by excitation of neurones bearing GluR5 receptors on their soma and/or dendrites, I looked at the effect of ATPA on mEPSCs in the presence of TTX (1 µM), to block action potential-dependent release. The IEI of mEPSCs in control was 359 ± 91 ms. This decreased to 262 ± 82 ms in the presence of ATPA (0.1 µM; KS test – $P<0.0001$; n=4; Figure 4.1D) reflecting an increase in frequency from 3.4 ± 0.9 Hz to 5.2 ± 1.5 Hz. The effect of ATPA was completely reversed by UBP 302 (20 µM). IEI increased from 262 ± 82 in the presence of ATPA to 366.55 ± 111.59 ms after subsequent addition of UBP 302 (KS test – $P<0.0001$; n=4; Figure 4.1D). Neither ATPA nor UBP 302 had any effect on amplitude or kinetics of the events (Table 4.1). Thus, the increase in frequency induced by ATPA seems likely to be activation of GluR5 containing receptors on the glutamate terminals, rather than on cell bodies where activation would lead to an increase in action potential-dependent release.

UBP 302 is non-selective in that it targets any KAr containing GluR5 subunits. In further experiments I examined the effect of NS 3763, an antagonist that is selective for homomeric GluR5 receptors (Christensen *et al*., 2004). In 3 neurones, ATPA decreased IEI from 604 ± 183 ms to 162 ± 65 ms (KS-test – $P<0.00001$; n=3), reflecting a change in frequency from 2.0 ± 0.6 Hz to 8.0 ± 2.4 Hz. Subsequent addition of NS 3763 (20 µM) increased IEI to 409 ± 145 ms (3.2 ± 1.2 Hz; KS-test – $P<0.0001$; n=3). However, IEI after addition of NS 3763 is still significantly increased from control (KS test – $P<0.00001$; n=3). NS 3763 had no significant effects on amplitude or kinetics of mEPSCs (Table 4.1). Thus, the studies with antagonists suggest that the presynaptic autoreceptor are likely to be a mixture of GluR5 homomers and heteromers in combination with another KAr subunit.

		IEI (ms)	Amplitude (pA)	Decay time (ms)	Rise time (ms)
sEPSCs	Control	174 ± 32	10.6 ± 0.7	5.8 ± 0.6	1.5 ± 0.1
	+ ATPA (0.1 µM)	80 ± 7*	11.4 ± 0.7	5.4 ± 0.5	1.4 ± 0.1
	+ ATPA (0.5 µM)	60 ± 9*^	11.3 ± 0.4	5.3 ± 0.5	1.4 ± 0.2
mEPSCs	Control	359 ± 91	12.3 ± 0.6	5.9 ± 0.2	1.5 ± 0.1
	+ ATPA (0.1 µM)	262 ± 82*	12.7 ± 0.8	5.5 ± 0.5	1.6 ± 0.1
	+UBP 302 (20 µM)	367 ± 112†	13.3 ± 1.4	5.5 ± 0.3	1.7 ± 0.1
	Control	604 ± 183	8.8 ± 1.3	6.7 ± 0.9	1.7 ± 0.2
	+ ATPA (0.1 µM)	163 ± 65*	10.0 ± 1.2	7.1 ± 0.3	1.4 ± 0.1
	+ NS 3763 (20 µM)	409 ± 145*†	8.6 ± 1.6	5.5 ± 0.6	1.7 ± 0.2

Table 4.1. Effects of GluR5 agonists/antagonists on IEI, amplitude and kinetics of sEPSCs and mEPSCs. **indicates a significant change when compared control (*P *<0.001 KS Test).* † *indicates a significant change when compared to 0.1 µM ATPA (*P *<0.001 KS Test).*

4.3.1.2. Effects of UBP 302 on sEPSCs

The next question I addressed was whether spontaneously released glutamate could tonically activate the presynaptic GluR5 receptors. To examine this I tested the effect of UBP 302 alone in 10 neurones. IEI in control conditions was 256 ± 512 ms. This remained unaltered in the presence of UBP 302 (20 µM; 274 ± 56 ms). Likewise, there was no change in amplitude or kinetics of events (Table 4.2). Thus, these experiments suggest that ambient glutamate release does not activate presynaptic KAr. In addition, the lack of effect of UBP 302 alone (see also Section 3.3.1.5.) suggests that GluR5-containing KAr do not contribute postsynaptically to sEPSCs, which are likely to be exclusively AMPAr-mediated.

		IEI (ms)	Amplitude (pA)	Decay time (ms)	Rise time (ms)
sEPSCs	control	256 ± 52	15.5 ± 3.4	5.2 ± 0.6	1.7 ± 0.2
	+ UBP 302 (20	274 ± 56	16.1 ± 1.6	5.9 ± 0.9	1.7 ± 0.1

Table 4.2. Summary of IEI, amplitude and kinetics of sEPSCs with UBP 302 alone.

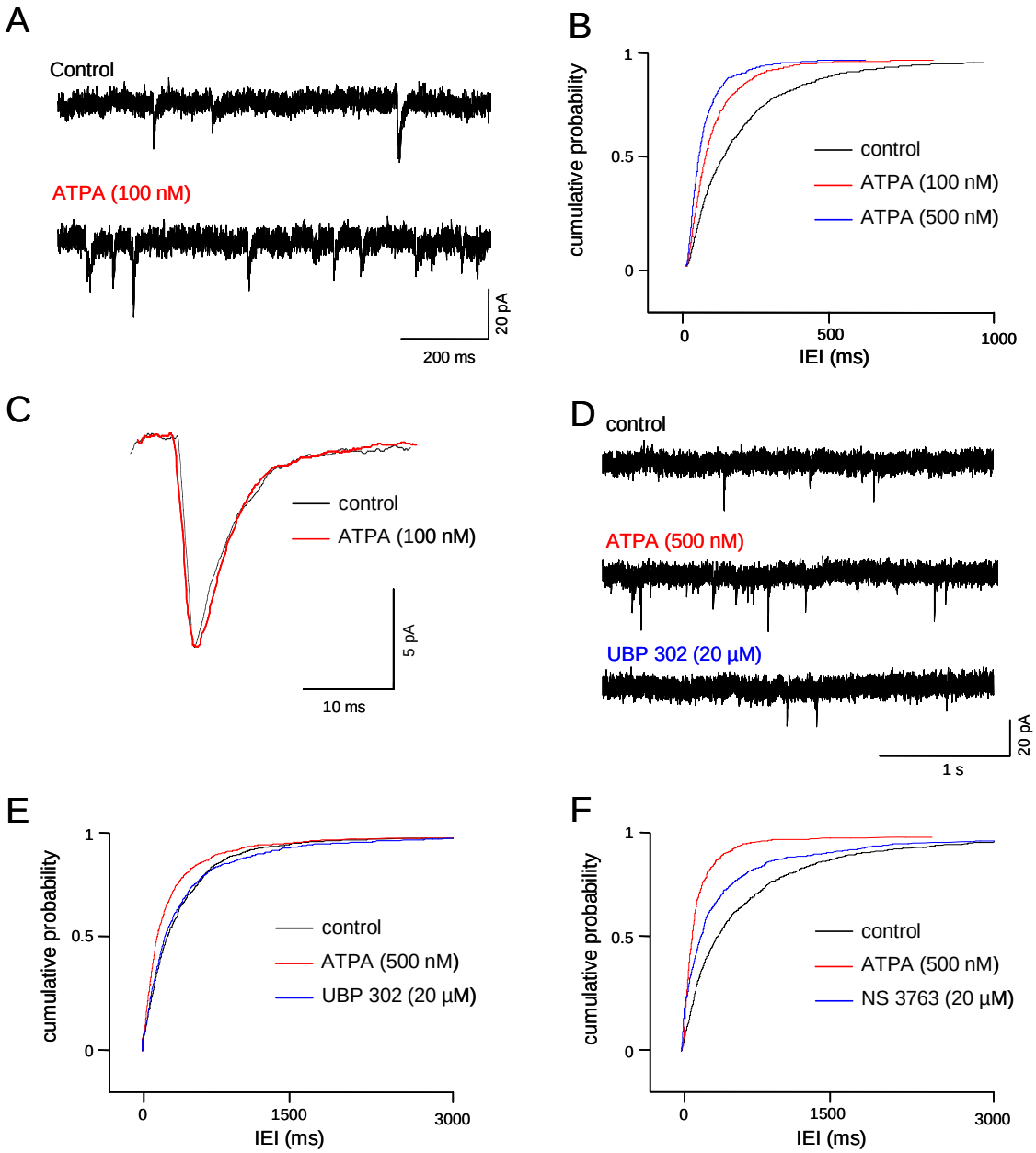


Figure 4.1. Effects of APTA on sEPSCs and mEPSCs. *A: Concurrent recordings of sEPSCs recorded in control conditions and during perfusion of ATPA (100 nM) in a typical layer III neurone. B: Cumulative probability graph of sEPSC IEI in control conditions and during ATPA (100 and 500 nM) application (n=7). C: Average sEPSC for control and after application of ATPA (n=60). D: Concurrent recordings of mEPSCs recorded in control conditions and during perfusion of ATPA (500 nM) and UBP 302 (20 µM). E: Cumulative probability graph mEPSC IEI in control conditions and during ATPA and UBP 302 application (n=4). E: Cumulative probability graph of mEPSC IEI in control conditions and during ATPA and NS 3763 (20 µM) application (n=3).*

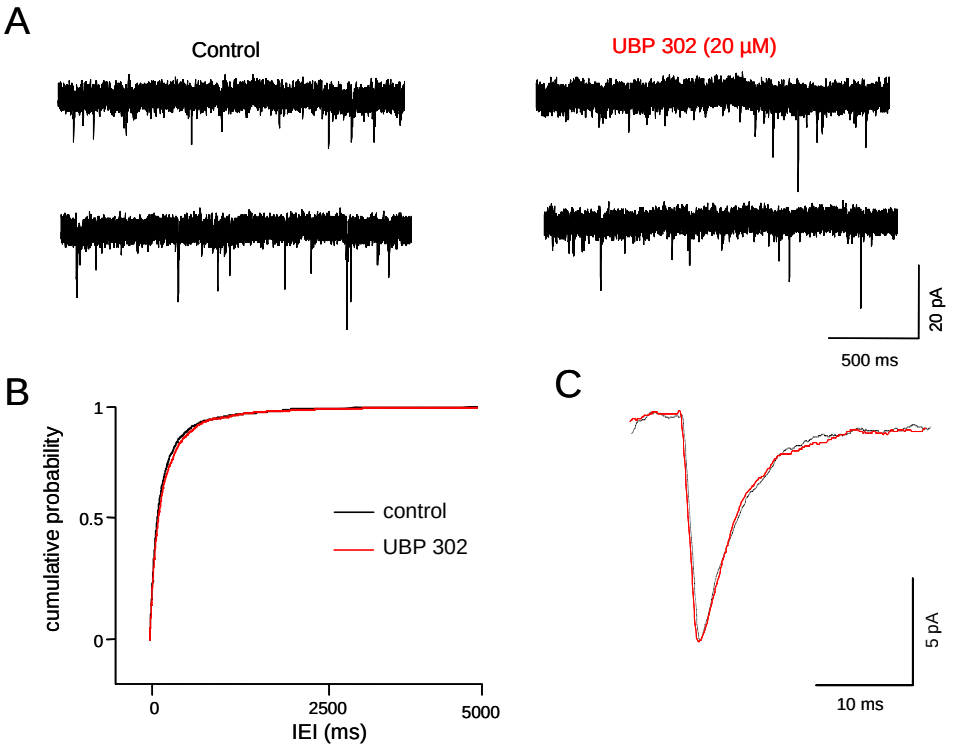


Figure 4.2. Effects of UBP 302 on sEPSCs. *A: Concurrent recordings from 2 example layer III neurones of sEPSCs in control conditions and during UBP 302 (15 µM) perfusion. B: Cumulative probability graph of sEPSC IEI in control conditions and after UBP 302 application (n=10). C: Averaged sEPSC in control conditions and after application of UBP 302 (n=60).*

4.3.1.3. eEPSCs in layer III

Using a bipolar stimulating electrode positioned in layer V of the lateral EC a train of EPSCs could be evoked in layer III of the medial EC. In these experiments I evoked trains of responses with 5 shocks at 5, 10 or 20 Hz. In all stimulus protocols, paired pulse facilitation (PPF) could be seen between the first and second shocks. With the stimulation frequencies employed the inter-pulse intervals were 200, 100 and 50 ms at 5, 10 and 20 Hz respectively. The paired pulse ratio seen with these frequency/intervals were constant at 1.3 ± 0.1 (n=4), 1.3 ± 0.1 (n=6) and 1.3 ± 0.2 (n=6). With 5 Hz stimulation, facilitation remained relatively constant across the train. When 10 Hz was used response amplitudes became more variable but in some cells, facilitation was increased. In contrast, at 20 Hz, amplitudes of later responses in the train decreased until they reached a plateau of approximately half the amplitude of the first response. Graphs showing the normalised average peak amplitude for pooled data

are shown in Figure 4.3B. eEPSCs were abolished by SYM 2206 (20 µM), indicating they are mediated by AMPAr (Figure 4.3A).

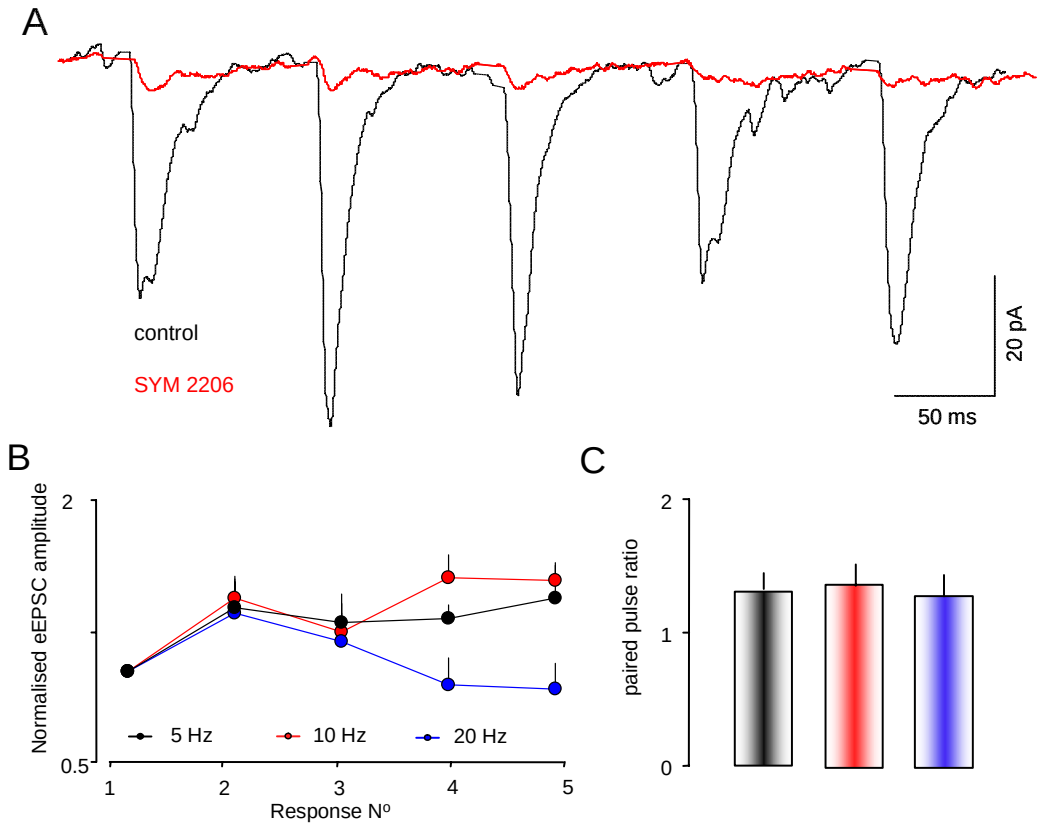


Figure 4.3. Evoked EPSCs in layer III of the EC. *A: Average trace of responses to trains of 5 shocks at 10 Hz recorded from an example cell in layer III in control conditions and after addition of SYM 2206 (20 µM). B: Graph of normalised amplitudes of responses to trains of 5 shocks for 5, 10, 20 Hz. C: Graph of PPR at different frequencies.*

4.3.1.4. Effects of ATPA on eEPSCs

The effects of ATPA and UBP 302 on sEPSCs suggested the presence of a GluR5-containing facilitatory KA autoreceptor on glutamate terminals in layer III. To look at this further I have tested the effect of ATPA (50 nM) on eEPSCs (5 pulses at 10 Hz; n=7; Figure 4.3A). As noted previously PPF of the amplitude of the second response in the train was observed under control conditions with peak amplitude increasing from 58.1 ± 11.6 pA to 67.3 ± 9.6 pA (P<0.01, n=7). Subsequent responses showed a gradual increase in facilitation (Figure 4.4). After the addition of ATPA (50 nM) the amplitude of the second response increased to 89.4 ± 20.3 pA, whilst the first response remained about the same (63.3 ± 16.6 pA; Figure 4.4B). The ratio of the paired pulse facilitation before and after the addition of ATPA (50 nM) increased from 1.2 ± 0.1 to 1.6 ± 0.2 (P<0.05, n=7). Subsequent perfusion with a higher

concentration of ATPA (100nM) greatly increased all responses in the train with the first and the second responses increased to 102.4 ± 21.4 pA and 105.3 ± 19.6 pA respectively. This meant that PPF was essentially eliminated and subsequent frequency-dependent facilitation throughout the train essentially disappeared (Figure 4.4C). ATPA had no effect on decay times of any of the evoked responses (Table 4.3). These data supported the results from the analysis of sEPSCs in the proceeding sections and indicate the presence of GluR5 autoreceptors facilitating glutamate release. They also suggest that the receptors may be largely saturated at 100 nM as responses no longer showed frequency-dependent facilitation.

	1	2	3	4	5
control	5.9 ± 0.7	6.9 ± 0.2	6.7 ± 0.5	6.7 ± 0.8	6.2 ± 0.4
+ ATPA	6.2 ± 0.9	6.3 ± 0.8	6.4 ± 0.4	6.6 ± 0.4	7.5 ± 1.0

Table 4.3. Effects of ATPA on decay times of eEPSCs in a stimulus train of 5 shocks at 10 Hz.

'Before and after' analysis of sEPSCs was also conducted. There were no significant differences in the amplitude or kinetics for events recorded before or after the trains in either control conditions or after addition of ATPA (100 nM). However, under control conditions the number of events increased from 7 ± 1 before the train 9 ± 2 after each train (P<0.05, n=7). In the presence of ATPA, the number of events before the train increased to 9 ± 2 (P<0.001, n=7) in agreement with the general increase in sEPSC frequency described earlier. Now, however, the number of events after the train did not change (9 ± 2; Figure 4.4D,E). Therefore the increased frequency of sEPSCs before the train in the presence of ATPA obscured the rise resulting from endogenous activation of the KAr on the terminals seen in control. These results suggest that the glutamate released during the stimulation trains may transiently increase sEPSC frequency, possibly as a result of KAr activation on the terminals. During ATPA application the change was obscured suggesting that the GluR5 receptors on the terminals were not fully activated and thus, unable to support further increase.

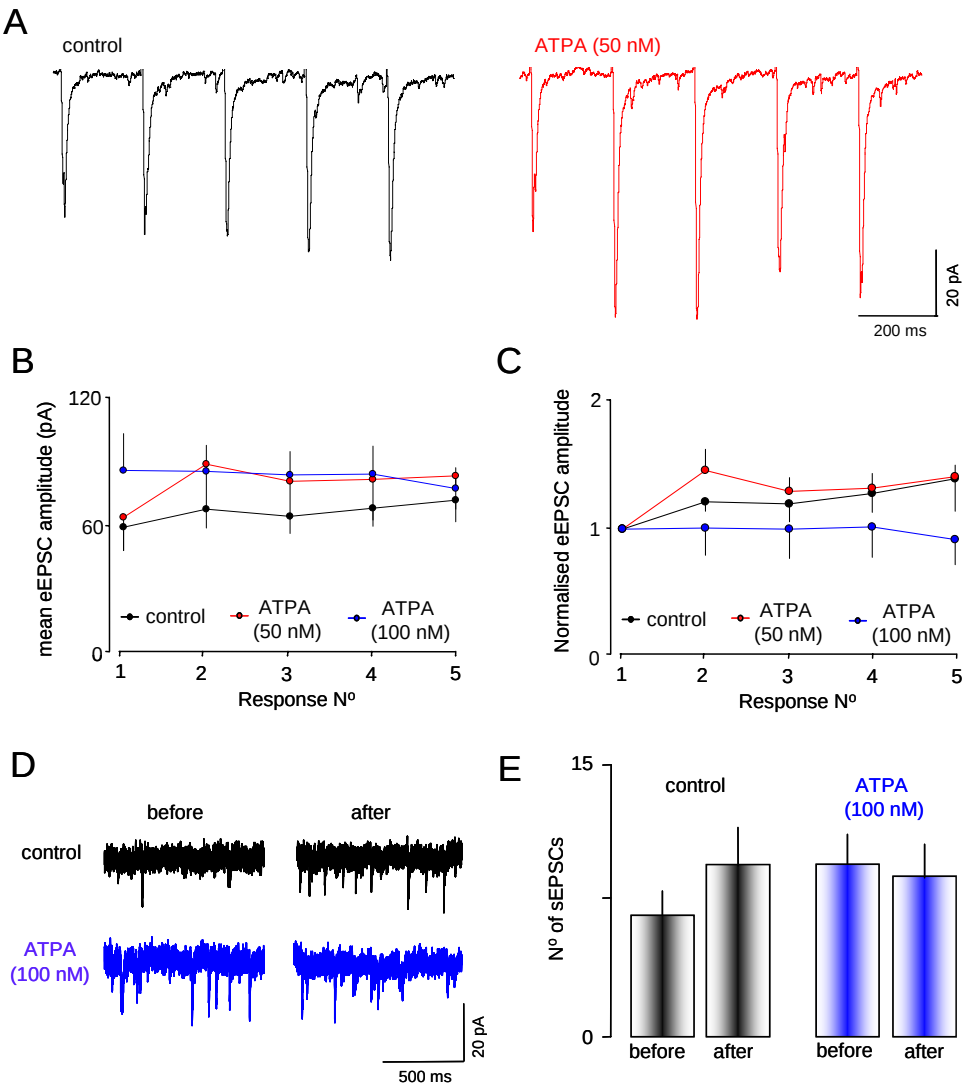


Figure 4.4. Effects of ATPA on eEPSCs. *A: Consecutive eEPSCs during 5 Hz train in control conditions and during perfusion of ATPA. B: eEPSCs mean amplitude graph in control conditions and after ATPA (50 and 100 nM) application (n=7) C: Graph of normalised amplitude of eEPSCs in control conditions and after ATPA application (n=7) D: Consecutive recordings of sEPSCs before and after stimulus train (5 Hz) in control conditions and after application of ATPA (100 nM) E: Graph of mean number of events before and after a stimulus train in control conditions and after perfusion of ATPA (n=7).*

4.3.1.5. Effects of UBP 302 on eEPSCs

Having shown that exogenous activation of GluR5 receptors could presynaptically enhance spontaneous and evoked release, in the next set of experiments I examined whether these receptors may be activated by endogenous glutamate release. The failure of UBP 302 to alter the frequency of sEPSCs (Section 4.3.1.2.) suggests that ambient glutamate release does not activate the receptors. To look at this further, I now tested the effect of the antagonist on eEPSCs. In these experiments I used 20 Hz

trains for 1s, in order to gain a picture of how frequency-dependent changes in eEPSCs were altered over a longer time course.

Again PPF of the amplitude of the second response in the train was observed with an average increase from 84.6 ± 36.0 pA to 102.3 ± 36.7 pA in 6 neurones (t-test – $P<0.05$, n=6). The facilitation persisted for the first 3-4 events in the train and was then succeeded by depression (Figure 4.5B). After the addition of UBP 302 (20 μM) the amplitude of first response was unaltered (82.8 ± 26.4 pA). However, the amplitude of the second response was now unchanged (79.5 ± 21.2 pA; Figure 4.5B) rather than facilitated. The decrease in PPR of the first two responses comparing control to UBP 302 was found to be statistically significant (t-test – 1.5 ± 0.2 to 0.9 ± 0.1, $p<0.05$, n=6). Amplitudes of later responses in the train decreased until they reached a plateau of approximately half the amplitude of the first response in all conditions (average of last ten responses in control – 51.8 ± 24.5; UBP 302 – 42.4 ± 17.1). Thus, UBP 302 effectively removed the early facilitation without altering the subsequent depression of eEPSCs. However, the antagonist had no effect on the kinetics of any of the evoked responses (average decay time in control – 7.3 ± 0.4 ms; UBP 302 – 6.8 ± 0.3 ms). Thus, the blockade of facilitation by UBP 302 clearly suggests that facilitation of glutamate release is dependent on a presynaptic GluR5 KAr, in line with the conclusions from the previous studies.

'Before and after' analysis with UBP 302 also supported this conclusion. In control conditions the number of events that occurred 1 s before the train of eEPSCs increased from 8 ± 2 to 11 ± 2 in the 1 s after (t-test – $P<0.05$, n=10). The addition of UBP 302 (20 μM) had no effect on the number of events before the train (7 ± 2), whereas the number of events after was no longer increased (7 ± 1, t-test – $P<0.01$, n=10; Figure 4.5D, E). There was no difference in the amplitude or kinetics for events recorded before or after the trains in either control or after addition of UBP 302. This analysis indicates that the GluR5 KAr autoreceptor is activated during repetitive stimulation and results in a subsequent brief increase in spontaneous events.

Overall, the conclusion is that GluR5 autoreceptors can facilitate glutamate release but are not tonically activated by ambient glutamate levels. During high frequency activation of glutamate synapses, sufficient glutamate is available to access the receptors and boost transmission.

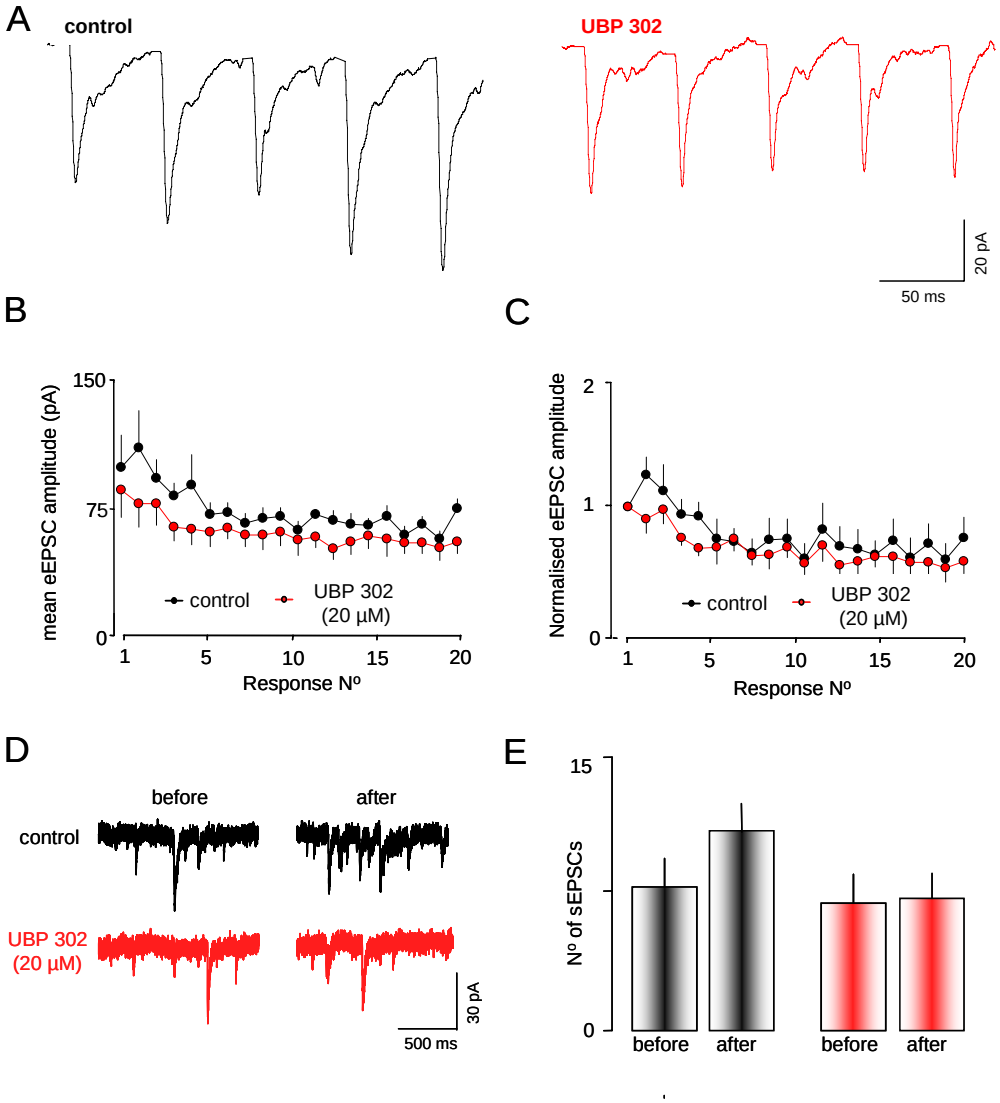


Figure 4.5. Effects of UBP 302 on eEPSCs. *Consecutive eEPSCs during 5 Hz train in control conditions and during perfusion of UBP 302 (20 µM). B: sEPSC mean amplitude graph in control conditions and after UBP 302 application (n=6) C: Graph of normalised amplitude of eEPSCs in control conditions and after UBP 302 application (n=6). D: Consecutive recordings of sEPSCs before and after stimulus train (20 Hz) in control conditions and after application of UBP 302. E: Graph of mean number of events before and after a stimulus train in control conditions and after perfusion of UBP 302 (n=6).*

4.3.1.6. Effects of 2-AP5

The KAr mediated facilitation of AMPAr-mediated eEPSCs seen in these experiments is reminiscent of the presynaptic NMDAr-mediated facilitation of glutamate release seen in layers II and V (Berretta and Jones, 1996b; Woodhall *et al*., 2001a). I thought it worthwhile therefore to test the effects of the NMDAr antagonist, 2-AP5 (50 µM) on eEPSCs evoked within layer III (5 pulses at 10 Hz). Again, facilitation of the

amplitude of the second response in the train was observed, the average increase was from 52.2 ± 6.1 A to 71.6 ± 7.21 pA in these neurones (KS test – $P<0.001$, n=5). In this particular group of neurones facilitation was maintained for all 5 shocks. However, after addition of 2-AP5, whilst the amplitude of first response remained the same (53.7 ± 6.5 pA) the amplitude of the second response was again decreased to 52.0 ± 5.8 pA (Figure 4.6A) with a consequent decrease in paired pulse ratio (1.5 ± 0.1 to 1.0 ± 0.1, t-test – $p<0.01$, n=5; Figure 4.6B). There was no change in kinetics of any of the evoked responses in the presence of 2-AP5. Subsequent addition of UBP 302 (20 µM) had no effect on the amplitude of the first response (50.5 ± 7.9 pA) and no further effect on the amplitude of the second response in the train (47.4 ± 4.9 pA) and therefore on PPR (1.1 ± 0.1). However, later responses in the train still showed some facilitation in the presence of 2-AP5 and this appeared to be blocked by UBP 302. These results were very surprising as they indicated that blockade of either the presynaptic GluR5 receptor or an NMDA autoreceptor was capable of completely preventing facilitation.

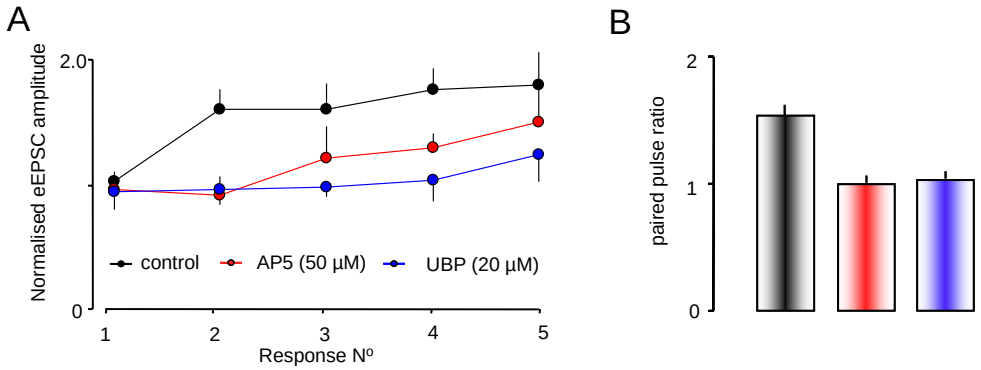


Figure 4.6. Effects of 2-AP5 on eEPSCs. *A: Graph of normalised amplitude of eEPSCs in control conditions and after 2-AP5 (50 µM) and UBP 302 (20 µM) application (n=5) B: Graph showing PPR in control conditions and during 2-AP5 and UBP 302 perfusion (n=5).*

As mentioned above, presynaptic NMDAr are tonically activated by spontaneous glutamate release in layers II and V (Berretta and Jones, 1996b; Woodhall *et al*., 2001a). However, this was not the case in layer III. Analysis of sEPSCs showed that there was no difference in frequency of sEPSCs, as shown by IEI (from 83.8 ± 13.4 to 87.7 ± 13.5; Figure 4.7A), amplitude or kinetics (Figure 4.7B). However, the increase in sEPSCs seen after evoked release revealed by before and after analysis was again dependent on NMDAr activation. Under control conditions, the number of events increased from 9 ± 1 before the train to 14 ± 1 after (t-test – $P<0.01$, n=10). With the addition of 2-AP5 the number of events before the train was the same as in control

conditions (8 ± 1) whereas the number of events after the train did not increase (9 ± 1, t-test – $P<0.01$, n=10; Figure 4.7C). There was no difference in the amplitude or kinetics for these events in the presence of 2-AP5. Subsequent addition of UBP 302 (20 µM) had no effect on the number of events before the train (9 ± 1) and no further effect on the number of events after the train (9 ± 1).

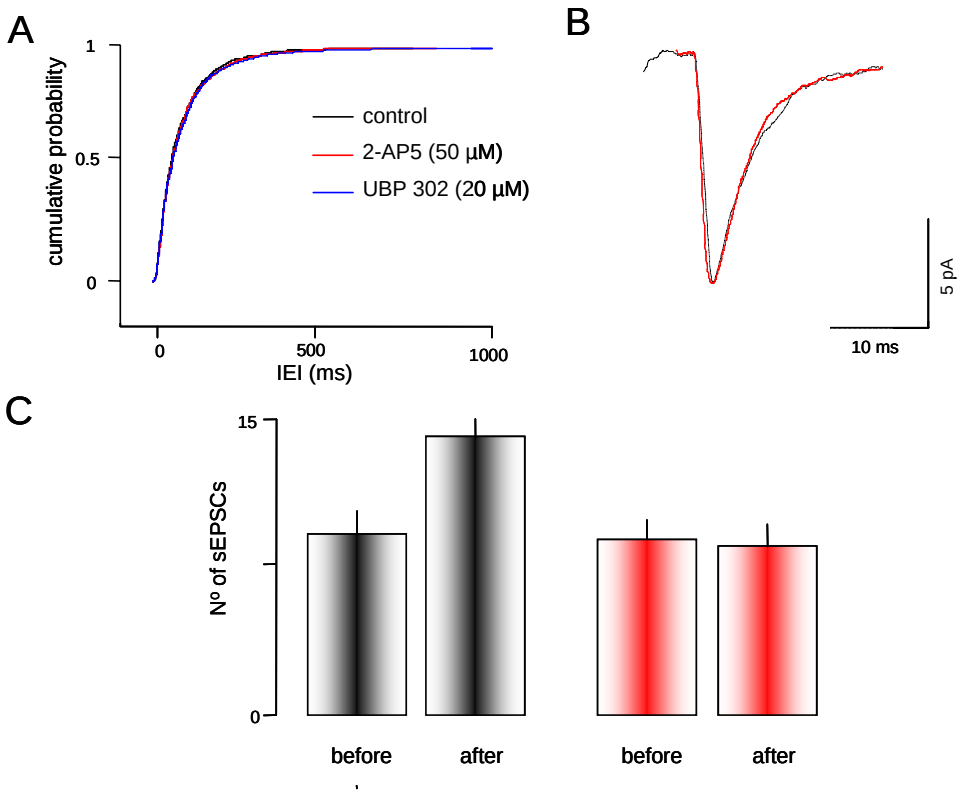


Figure 4.7. 2-AP5 on sEPSCs. *A: Cumulative probability graph of sEPSC IEI in control conditions and after 2-AP5 (50 µM) and UBP 302 (20 µM) application (n=10) B: Averaged sEPSCs in control and 2-AP5 (n=60). C: Graph showing mean number of events before and after a stimulus train in control conditions and after perfusion of 2-AP5 (n=10).*

One possible explanation for the above results is that UBP 302 may be non-selective and act as an NMDAr antagonist. To ensure that UBP 302 was having no direct effects on the NMDAr I tested its effects on evoked NMDAr mediated responses in five neurones. The NMDA responses were isolated by blocking AMPAr, $GABA_Ar$ and $GABA_Br$ using GYKI 53655 (25µM), SR 95531 (20µM) and CPG 55845 (5µM) respectively, and recording at a holding potential of +40 mV. Single responses were evoked and six successive NMDAr-mediated eEPSCs for each neuron were averaged. The eEPSCs had an average amplitude of 138.7 ± 14.4 pA and slow kinetics (decay time, 158.2 ± 42.0 ms; 10-90 % rise time, 2.5 ± 0.8 ms; n=5). They could be abolished by 2-AP5 (50 µM), indicating they were indeed mediated by NMDAr. However,

application of UBP 302 (20 µM) had no effect on amplitude (126.6 ±12.5 pA), time to decay (182.1 ± 59.3 ms) or 10-90% rise time (3.3 ± 0.3 ms). Thus, it is unlikely that the effects of UBP 302 on facilitation in layer III results from NMDAr blockade.

I have also looked at the effects of 2-AP5 on eEPSCs and sEPSCs after the addition of ATPA. Addition of 50 nM ATPA increased PPF of evoked EPSCs from 1.14 ± 0.06 to 1.41 ± 0.09 (n=5) as described earlier (Section 4.2.1.4). However, subsequent addition of 2-AP5 had no effect (1.40 ± 0.09; Figure 4.8A,B). ATPA also decreased IEI of sEPSCs from 352 ± 117 ms to 128 ± 28 ms. Subsequent addition of 2-AP5 did increase IEI to 226 ± 60 ms (KS test – $P<0.001$, n=5) but was still decreased when compared with control (KS test – $P<0.01$; Figure 4.8C). There was no change in the amplitude or kinetics of these events in the presence of 2-AP5.

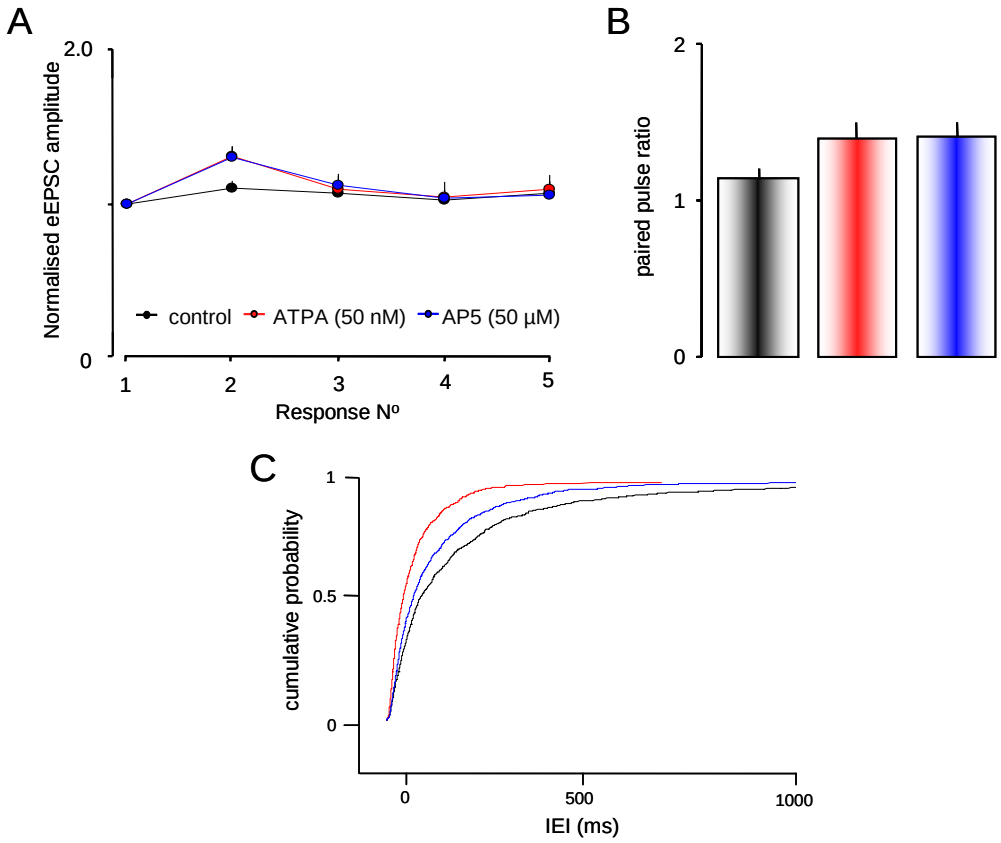


Figure 4.8. Effects of 2-AP5 on KAR mediated facilitation. *A: Graph of normalised amplitude of eEPSCs in control conditions and after ATPA (50 nM) and 2-AP5 (50 µM) application (n=5). B: Graph of PPR for control conditions and after application of ATPA and 2-AP5* (n=5). *C: Cumulative probability graph of sEPSC IEI in control conditions and after ATPA and 2-AP5 application (n=5).*

4.3.2. KAr at postsynaptic sites

The foregoing experiments have clearly indicated the presence of presynaptic GluR5 KA autoreceptors in layer III. Recently, West *et al*. (2007) described a KAr eEPSC at these synapses. I now sought to confirm if KAr contributed to the eEPSCs at these synapses and to gain some idea of the KAr subunits involved. At other synapses KAr EPSCs are hard to detect and rely on observing a residual current when AMPAr and NMDAr are blocked. I used this approach here. I blocked NMDAr, $GABA_A$r, $GABA_B$r and AMPAr receptor mediated currents using a cocktail of 2-AP5 (50 µM), SR 95531 (20µM), CPG 55845A (5µM) and either SYM 2206 (20 µM) or GYKI 53655 (25µM) and used a stimulus train of either 50, 100 or 200 Hz increase to elicit the KAr eEPSC. In the presence of these antagonists, single shock stimulation evoked very little in the way of an eEPSC. However, with high frequency trains a small, slow KAr-mediated current was recorded in 57% of cells (8/14) following high frequency stimulation.

4.3.2.1. Pharmacology of KAr eEPSC

The KAr eEPSC showed a clear dependence on frequency of activation (see Section 4.3.2.2 below). In my pharmacological analysis I used trains of 5 shocks at 100 Hz to evoke responses. In the presence of the antagonist cocktail there was a small, slow residual current. In the 8 neurones tested, the mean amplitude of the slow current was 22.5 ± 1.2 pA and its decay time was 232.8 ± 26.2 ms. In the presence of UBP 302 (20 µM) these parameters were unaltered at 20.3 ± 1.5 and 258.7 ± 26.3 ms (Figure 4.9). Subsequent addition of CNQX (10 µM) completely blocked the eEPSC. This concentration is relatively selective for KAr over AMPAr, but does not discriminate between KAr subtypes. These results confirm those of West *et al*. (2007). Thus, my data indicate that this eEPSC is not mediated by KAr containing the GluR5 subunit but is likely to depend on other KAr subunit / subunits.

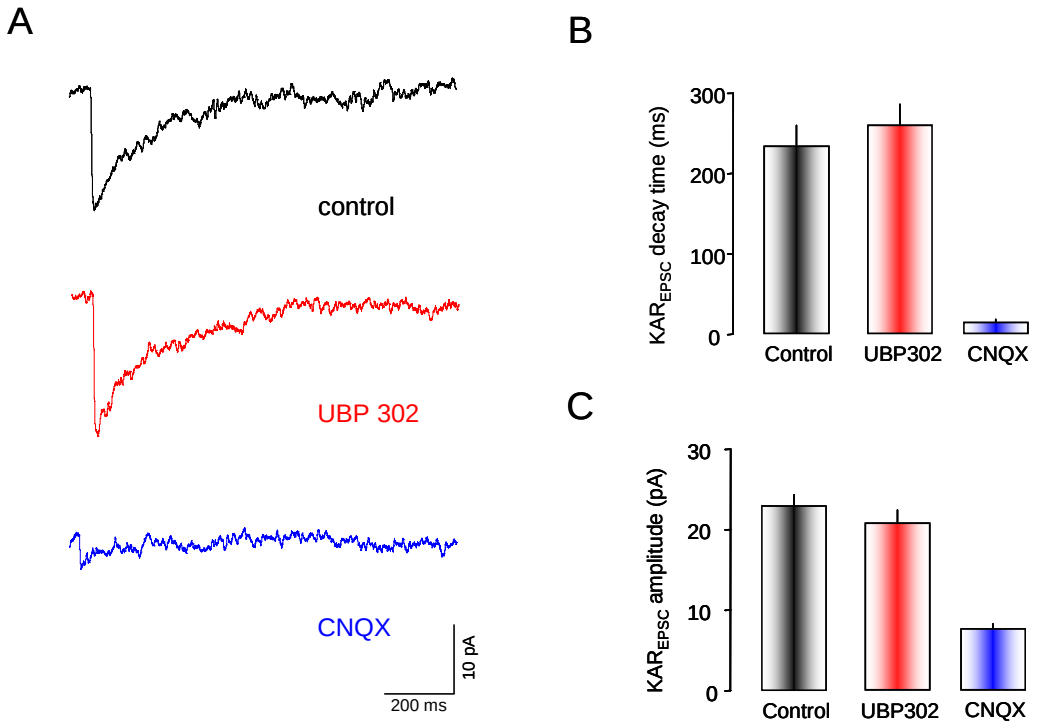


Figure 4.9. Pharmacology of KAr eEPSC *A: Consecutive recordings of KAr eEPSC in control conditions and in the presence of UBP 302 (20 µM) and CNQX (10 µM). B,C; Graphs showing mean decay time (B) and peak amplitude (C) of KAr-mediated eEPSCs in control conditions and after addition of UBP 302 and CNQX* (n=8).

4.3.2.2. Frequency-dependence of KAr eEPSC

The amplitude and decay time constants of the KAr EPSC were similar to those previously reported (West *et al*., 2007; Castillo *et al*., 1997). Both amplitude and decay time of the KAr eEPSC were dependent on the frequency and the duration of stimulus train.

Increasing the frequency from 50 to 100 Hz but keeping train length constant resulted in an increase in amplitude from 20.9 ± 1.4 pA to 24.3 ± 1.2 pA (n=4). Decay time concurrently rose from 132.9 ± 23.2 ms to 189.9 ms. Increasing the stimulation frequency to 200 Hz again maintaining the same duration of train further increased both amplitude (to 35.7 ± 5.4 pA) and decay time (to 378.1 ± 35.2 ms; Figure 4.10A). The increase in both amplitude and decay time was found to be significant only after the increase to 200 Hz ($P<0.05$; One way ANOVA and Bonferroni Test).

Similarly, increasing the length of the stimulus train but maintaining the frequency also increased the KAr eEPSC parameters. Thus, augmenting the number of shocks

from 5 to 10 at 50 Hz caused an increase in amplitude from 18.3 ± 1.5 pA to 25.5 ± 1.5 pA (ANOVA – $P<0.05$, n = 7) and in the decay time from 183.5 ± 29.8 to 298.2 ± 48.0 ms. 15 shocks at 50 Hz further increased the amplitude to 32.4 ± 2.3 pA (ANOVA – $P<0.05$, n = 7) and the decay time to 404.8 ± 61.5 ms (ANOVA – $P<0.05$; Figure 4.10B). Averages of the amplitude and decay times for all stimulation protocols are included in Table 4.4 and are illustrated in Figure 4.10.

	Amplitude (pA)	Decay time (ms)
50 Hz	20.9 ± 1.4	132.9 ± 23.1
100 Hz	24.3 ± 1.2	189.9 ± 19.8
200 Hz	35.7 ± 5.4*	378.1 ± 35.2*
5 shocks at 50 Hz	18.3 ± 1.5	183.5 ± 29.8
10 shocks at 50 Hz	25.5 ± 1.5*	298.2 ± 48.0
15 shocks at 50 Hz	32.4 ± 2.3*†	404.8 ± 61.5*

Table 4.4 – Summary of KAr eEPSC amplitude and decay times at different frequencies and durations of stimulus train. ** indicates a significant change when compared control (P <0.05 One way ANOVA and Bonferroni Test). † indicates a significant change when compared to 10 shocks (P <0.001 One way ANOVA and Bonferroni Test).*

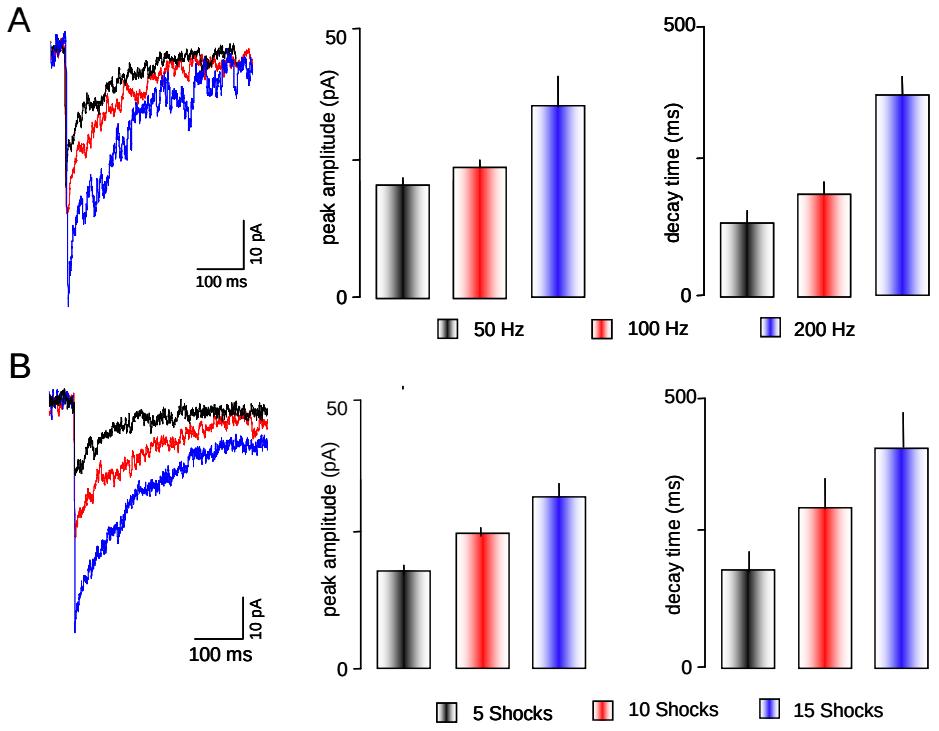
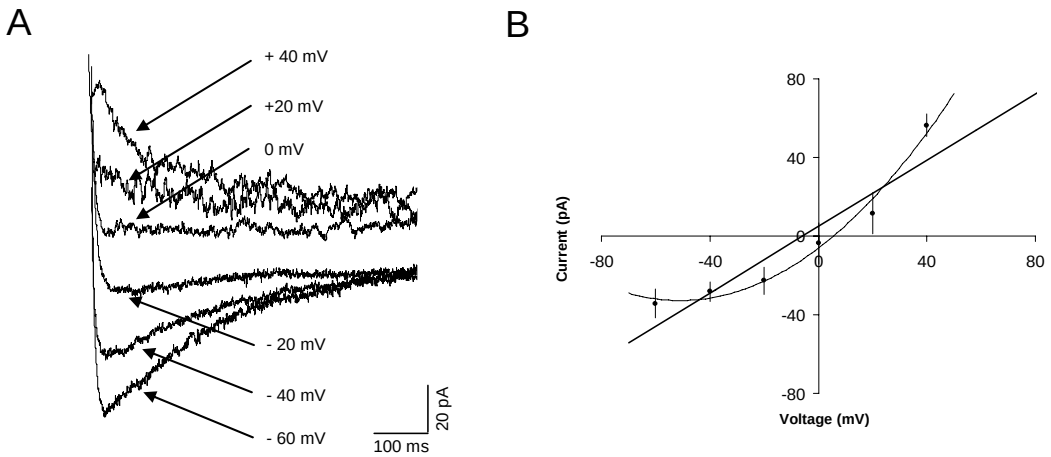


Figure 4.10. Frequency-dependence of KAr eEPSC *A: Concurrent recordings and graphs showing mean peak amplitude and decay time of KAr eEPSC after 50, 100 and 200 Hz stimulation. B: Concurrent recordings and graphs showing mean peak amplitude and decay time of KAr eEPSC after 5, 10 and 15 shocks.*

4.3.2.3. I-V relationships of KAr eEPSCs

The *I-V* relationship of KAr can reflect the calcium permeability and the subunit composition of channels (Egebjerg and Heinemann, 1993; Ruano *et al*., 1995). Inward rectification is characteristic of KAr containing unedited GluR5 and GluR6 subunits (Bowie and Mayer, 1995; Kamboj *et al*., 1995). Thus, I also studied the current-voltage (*I-V*) relationship of the KAr eEPSC. In the presence of the antagonist cocktail detailed above, KAr eEPSCs, induced by 100 Hz stimulation, were recorded at holding potentials ranging from –60 to +40 mV. KAr eEPSCs reversed at a potential of approximately 0 mV (-3.2 ± 4.7 mV, n = 4). The current recorded at the peak amplitude in relation to the holding potential was then plotted. Figure 4.9E illustrates the *I-V* curve (corrected for LJP) of KAr eEPSCs. It can be seen that the I-V relationship is relatively linear in the negative voltage range, but shows a pronounced inward rectification at positive holding potentials. Thus, this data combined with the observations that the eEPSC is blocked by CNQX and is unaltered by UBP 302 suggests that there is a small, prolonged EPSC in layer III neurones likely mediated by KAr containing unedited GluR6 subunits.



4.11. I-V relationship of KAr eEPSC. *A: Concurrent KAr eEPSCs recorded at holding potentials from – 60 to + 40 mV in an example layer III neurone. B: Current-Voltage plot (n=4; corrected for LJP).*

4.4. Discussion

These results provide strong electrophysiological evidence for the presence of two different types of KAr involved in glutamatergic neurotransmission in layer III of the EC. They confirm that layer III neurones receive excitatory inputs mediated by

postsynaptic KAr (West *el al*. 2007) but extend this to show that the receptors involved are not GluR5 containing, but may be homo- or heteromeric receptors containing the GluR6 subunit. In addition, I have shown that glutamate transmission is physiologically boosted by a KA autoreceptor, which is a homo- or heteromeric GluR5-containing receptor.

Frequency-dependent facilitation of AMPAr-mediated eEPSCs was blocked by UBP 302, the GluR5 selective antagonist, and enhanced by a low concentration of ATPA, the GluR5 selective agonist. Thus, it seems likely that this facilitation is mediated by a presynaptic KAr containing the GluR5 subunit. This conclusion was strongly supported by the observation that ATPA increased the frequency of sEPSCs without changing mean amplitude or kinetics. The lack of effect of UBP 302 on sEPSCs in these neurones indicates that the presynaptic GluR5 receptors are not tonically activated by spontaneous glutamate release and likely to be activated only in situations where higher levels of glutamate are present. This is shown by two observations. First during trains of AMPAr-mediated eEPSCs, the first event was unaltered by UBP 302, but the facilitation of succeeding events was blocked. Second, following a train of eEPSCs, the frequency of AMPAr mediated sEPSCs was transiently increased, and this effect was also blocked by UBP 302, without change in amplitude or kinetics of the spontaneous events. Thus, these studies provide evidence that the presynaptic KAR is activated during high levels of excitation, i.e. repetitive stimulation, and act to further boost excitation (see Figure 4.12).

The precise mechanism for the facilitation is currently unknown. However, it seems likely to be an ionotropic response, not metabotropic as has been suggested for presynaptic KAr previously (Rodriguez-Moreno and Sihra, 2004), due to the speed at which it occurs, although, the possibility of a metabotropic effect cannot be completely ruled out. If it is ionotropic, the mechanism probably involves increased influx of Ca^{2+} into the glutamate terminal. This could occur by two mechanisms. Activation of the GluR5 receptor could depolarise the terminal and cause increased opening of VGCCs, leading to enhanced glutamate release. Alternatively, increased Ca^{2+} entry could occur directly via the receptor ionophore, as KAr do have a low Ca^{2+} permeability (Ferrer-Montiel and Montal, 1996), with unedited GluR5 and GluR6 containing receptors having higher permeability than those that have undergone mRNA editing (Burnashev *et al.*, 1996). This has been proposed for facilitation by

presynaptic NMDAr (Woodhall *et al*., 2001a). We do not yet know what the situation is with the presynaptic KAr in layer III. However, it is clear that the receptor is localised to the terminals, as ATPA caused an increase in mEPSC frequency as well as its effects of action potential driven release. This shows that the increase in glutamate release is unlikely to be (solely) dependent on increased firing in afferent or recurrent excitatory connections.

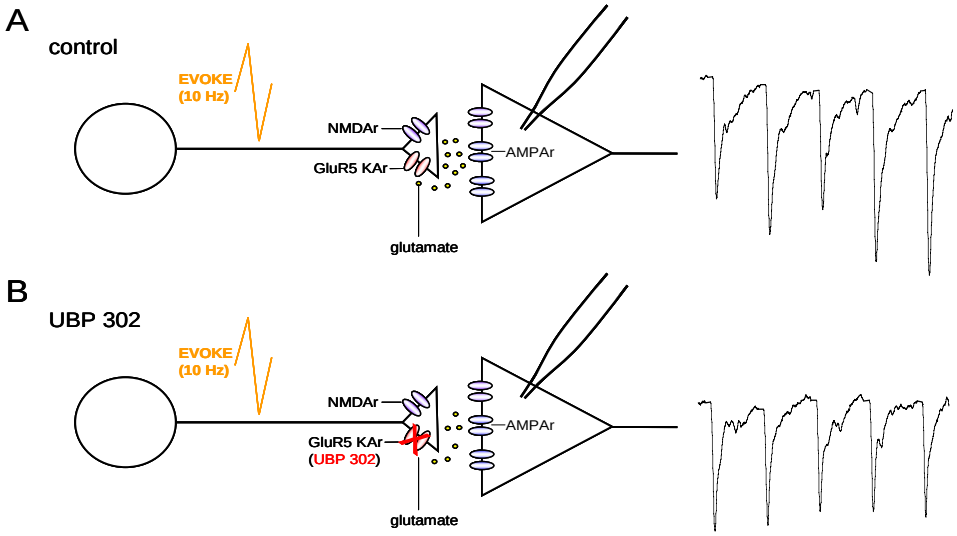


Figure 4.12. Schematic summary diagram of presynaptic KAr in glutamatergic transmission. *Schematic diagrams showing what may be happening during 10 Hz stimulation in control conditions (A) and after application of UBP 302 (B).*

Previous studies in the EC have shown that there are a number of other presynaptic receptors at glutamatergic synapses (Woodhall *et al*., 2007; Thompson *et al*., 2006; Evans *et al*., 2000; Woodhall *et al*., 2001a; Berretta and Jones, 1996b) controlling transmitter release. NMDA autoreceptors are present in layers II and V where they act to facilitate glutamate release, and in contrast to the presynaptic GluR5 KAr are tonically active (Berretta and Jones, 1996b; Woodhall *et al*., 2001a; Yang *et al*., 2006a).

Because the effects of the NMDAr and KAr in causing facilitation show similarities, I felt it was important to determine any role of presynaptic NMDAr in layer III. Initial experiments with 2-AP5 showed no effect on sEPSC frequency (see also Section 3.3.1.5.), suggesting that presynaptic NMDAr were not operative in layer III, in contrast to layers II and V. However, very surprisingly, I then found that 2-AP5 blocked frequency facilitation of AMPAr-mediated eEPSCs. Even more surprising,

after blocking NMDAr UBP 302 no longer had any effect and vice versa. The fact that UBP had no direct effect on the isolated NMDAr-mediated evoked EPSC ruled out the possibility that UBP 302 itself is blocking NMDAr.

These observations are difficult to explain. One possibility is that presynaptic KAr and NMDAr are acting synergistically on some terminals to facilitate glutamate release in layer III. However, this may only be the case during low levels of KAr activation, because when facilitation was increased by ATPA, blocking NMDAr no longer blocked the frequency facilitation. In the presence of ATPA, 2-AP5 did decrease sEPSC frequency slightly, although it was still much higher than that seen in control. It may be speculated that during repetitive stimulation, activation of the presynaptic KAr causes the necessary depolarisation of the terminal but not enough to facilitate glutamate release directly. However, the depolarisation of the terminal could then result in parallel activation of the presynaptic NMDAr by relieving Mg^{2+} block. These experiments with ATPA suggest that the KA autoreceptor is capable of causing facilitation without synergy with the NMDAr, if the levels of available glutamate are sufficiently high. At present then the basis of the KAr-NMDAr synergy remains unknown and the subject for future investigation.

Positive feedback of the nature mediated by the KA autoreceptor described here could be considered a dangerous feature as it introduces inherent instability to a synapse. Such instability could possibly underlie the susceptibility of the EC to epileptiform activity, and in particular the vulnerability of layer III to degeneration in patients and animal models of TLE (Du and Schwarcz, 1992; Du *et al*., 1993; Du *et al*., 1995). As mentioned in Chapter 1, KA's ability to cause acute epileptiform activity and a chronic epileptic condition, where cell loss seen is also similar to that seen in patients with TLE (Du *et al*., 1995), suggests that KAr may be possible future targets for pharmacological interventions. More recently, there has been direct evidence for the involvement of GluR5 subunit containing KAr in epilepsy, as antagonists of GluR5 (LY377770 and LY382884) prevent pilocarpine induced limbic seizures (Smolders *et al*., 2002) and topiramate, an antiepileptic drug whose action was previously unknown, has been found to protect against seizures induced by ATPA (Kaminski *et al*., 2004). There has been a suggestion that GluR5 agonists could be used antiepileptic drugs by virtue of the fact that they can increase GABA release (Khalilov *et al*., 2002; see also Section 5.3.1.1.). However, the evidence I have presented for

GluR5-containing KAr producing a significant increase in excitation may directly challenge this idea. I have clearly demonstrated that, in layer III, a presynaptic GluR5 containing KAr is capable of greatly increasing excitation, and therefore a GluR5 agonist may exacerbate epilepsy in this region. It is noteworthy that, in these experiments, inhibition was not blocked and therefore this action of the KAr occurred regardless of whether there is also a GluR5 KAr acting to increase inhibition in the EC, although this will be discussed in more detail in Chapter 5.

In addition to the facilitatory presynaptic GluR5 KAr, KAr also mediate postsynaptic effects in the EC. A KAr-mediated synaptic current has been identified in the EC by West *et al*. (2007). In the present study, neither UBP 302 nor ATPA had any effect on the average kinetics or amplitude of sEPSCs suggesting that the GluR5 subunit containing KAr did not contribute to the postsynaptic glutamate currents in layer III. However, KAr-mediated evoked synaptic currents could be isolated, when AMPAr, and NMDAr were blocked and repetitive stimulation was employed, an approach used by West *et al*. (2007) and others to study postsynaptic KAr responses. The KAr mediated eEPSC in my studies was very similar to that found by West *et al*. (2007). Most importantly time to decay, which is often used to separate KAr-mediated currents from much faster AMPAr-mediated currents, was very similar. The slow eEPSC could also be blocked by CNQX at concentrations selective for KAr. However, UBP 302 had no effect on this KAr-mediated eEPSC in my experiments. This clearly shows that the postsynaptic KAr responsible for the eEPSC does not contain the GluR5 subunit. In addition, the I-V curve for the KAr eEPSC showed significant inward rectification. The I-V relationship of KAr can reflect the calcium permeability and the subunit composition of channels (Egebjerg and Heinemann, 1993; Ruano *et al*., 1995). Inward rectification is characteristic of KAr containing unedited GluR5 and GluR6 subunits (Bowie and Mayer, 1995; Kamboj *et al*., 1995) which are relatively impermeable to Ca^{2+}. Together, these data indicate that the KAr responsible for these eEPSCs in layer III contain an unedited GluR6 subunit (Figure 5.13.)

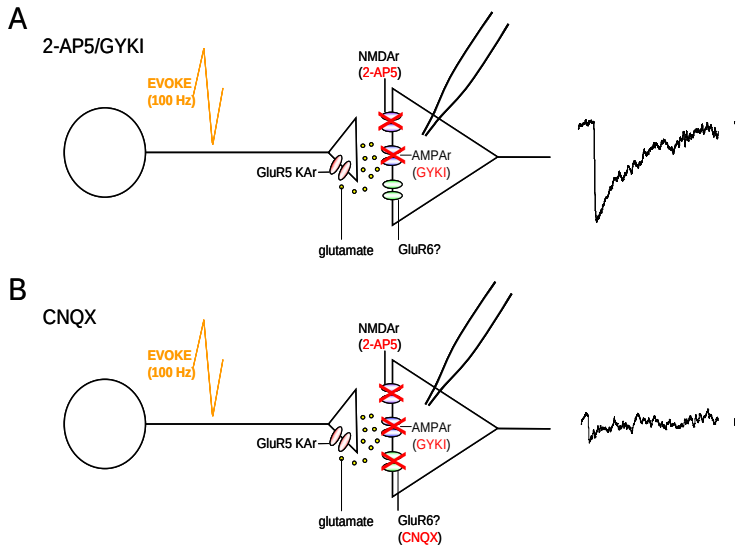


Figure 4.13. Schematic summary diagram of postsynaptic KAr on principal neurones in layer III. *Schematic diagrams showing what may be happening during 100 Hz stimulation in 2-AP5 and GYKI 53665 (A) and after application of CNQX (B).*

As discussed in Section 1.5.5., since KAr mediated synaptic transmission was first identified by (Castillo *et al*., 1997), it has been found throughout the brain (Frerking *et al*., 1998; DeVries and Schwartz, 1999; Kidd and Isaac, 1999; Li *et al*., 1999; Ali, 2003; Gryder and Rogawski, 2003). A recent study in the anterior cingulate cortex in genetically modified mice showed that both GluR5 and GluR6 subunits contribute to the KAr EPSCs, with GluR6 being more prominent (Wu *et al*., 2005). As double knockouts completely abolished the current the authors concluded that it was unlikely that GluR7 was involved. We have no evidence to show which subunits are responsible for the postsynaptic KAr mediated current in the EC, only that GluR5 does not appear to be involved, and they may contain an unedited GluR6 subunit. All of the KAR subunits are expressed in detectable levels (with exception of KA1) in the adult rat EC (Bahn *et al*., 1994), so any of them may be involved. As yet, the only subunit-selective ligands available are those for GluR5, therefore in order to determine which subunits are involved in the postsynaptic current in the EC either genetic knockouts must be tested or studies must wait for development of further useful antagonists.

Importantly, the present study provides evidence for at least two KAr with different subunit compositions contributing to transmission at glutamatergic synapses in layer III of the EC, a presynaptic receptor acting to facilitate glutamate release, which contains the GluR5 subunit, and a postsynaptic receptor that contributes to synaptic

transmission but does not contain the GluR5 subunit. This suggests that, at least in the EC, KAr with different functions have different subunit compositions. Thus, the susceptibility of the EC to epileptogenesis and neuronal damage induced by KA, will likely involve enhanced glutamate release, coupled with direct activation of the postsynaptic receptors. This leads to the conclusion that therapeutic interventions for TLE may be most effective if they are selective for to receptors composed of specific subunits. Also, as subunit expression of KAr is thought to be altered in epilepsy (Porter *et al*., 2006), it will be of great interest to determine if there are obvious changes in KAr in the EC in animal models of TLE.

Finally, the present results are of relevance to the generation of oscillatory activity in the EC. Both gamma oscillations (Cunningham *et al*., 2003; Stranger *et al*., 2008) and SWO (Cunningham *et al*., 2006) generation appear to involve GluR5 KAr. It is possible that exacerbation of glutamate transmission driven by GluR5 autoreceptors, together with a high degree of recurrent connectivity in layer III (Dhillon and Jones, 2001) could be a factor in susceptibility to rhythmical network activity.

CHAPTER 5

LOCALIZATION AND FUNCTION OF KAR AT GABAERGIC SYNAPSES IN LAYER III

5.1. Introduction

In the previous Chapter I showed that presynaptic KAr are present at excitatory synapses in layer III acting to facilitate glutamate release. Presynaptic KAr also exist as heteroreceptors at inhibitory synapses throughout the CNS (Rodriguez-Moreno and Lerma, 1998; Min *et al*., 1999; Cossart *et al*., 2001; Frerking *et al*., 2001; Braga *et al*., 2003; Crowder *et al*., 2006), where they have been show to both increase (Cossart *et al*., 2001) and decrease (Min *et al*., 1999) GABA release. Therefore, the aim of this chapter was to examine presynaptic KAr at GABAergic synapses, and to identify their role in layer III of the EC.

Early evidence for KAr regulating GABA release came from studies showing that low doses of KA caused a reduction in the amplitude of sIPSCs and eIPSCs in hippocampal pyramidal cells (Fisher and Alger, 1984; Kehl *et al*., 1984). However, this work did not really progress until selective ligands were developed to distinguish between the AMPAr and KAr. In 1997, Clarke *et al*. (1997) showed that the GluR5 selective KAr agonist, ATPA, caused a reduction in eIPSC amplitude in the hippocampus. In addition, the GluR5 selective KAr antagonist, LY294486, prevented the action of both ATPA and KA. In the same year, Rodriguez *et al*. (1997) showed that application of KA reduced the frequency of mIPSCs and the amplitude of eIPSCs, both in culture and hippocampal slices. These effects were blocked by CNQX. Since then KAr have been shown to inhibit GABA release at inhibitory synapses in other brain regions including in the globus pallidus (Jin and Smith, 2007) and in the amygdala (Braga *et al*., 2003).

Initially, the ability of KAr to depress GABA release was difficult to reconcile with the ionotropic nature of the receptor. However, Rodriguez *et al*. (1997) provided evidence for a metabotropic action of KAr by showing that the effects of KA were blocked by pre-incubation with pertussis toxin, or PKC inhibitors such as staurosporine and calphostin C. In terms of the subunit composition of the KAr responsible, some evidence for the presence of GluR6/7 has been shown in the globus pallidus using immunoreactivity (Jin and Smith, 2007), whilst pharmacological evidence suggests the involvement of GluR5 in the hippocampus and in the amygdala (Clarke *et al*., 1997; Braga *et al*., 2003). It is thought that the metabotropic actions of

KAr underlie their ability to depress GABA release (Rodriguez-Moreno and Sihra, 2007).

KAr have also been shown to facilitate GABA release at a number of inhibitory synapses throughout the CNS, including CA1 (Cossart *et al*. 2001), the hypothalamus (Liu *et al*. 1999), the neocortex (Ali at al., 2001), the dorsal horn of the spinal cord (Kerchner *et al*. 2001), the nucleus accumbens (Crowder *et al*., 2006) and in the amygdala (Braga *et al*. 2003). Studies of subunit composition suggest that there are no obvious differences in the KAr that facilitate GABA release compared to those that inhibit it. Using confocal and electron microscopy, Lu *et al*. (2005) showed that 20-35% of GAD67 positive terminals in the dorsal horn also show immunoreactivity for GluR5, GluR6/7 and KA1 and 2 KAr subunits. In CA1, use of subunit-specific knockouts have shown that the receptors facilitating GABA release are likely to be GluR5/GluR6 heteromers (Mulle *et al*., 2000).

As presented in Section 1.5.6., presynaptic KAr at inhibitory synapses (as at excitatory synapses) may modulate GABAergic transmission in a bidirectional manner, with lower concentrations of agonist causing an increase in transmitter release, and higher concentrations a depression. This has been shown in both CA1 and CA3 (Jiang *et al*., 2001; Schmitz *et al*., 2000; 2001) and in the amygdala (Braga *et al*., 2003). Interestingly, only one synapse has so far been identified, in the globus pallidus (Jin and Smith, 2007), where presynaptic KAr are capable of only depressing GABA release without bidirectional modulation.

In addition to presynaptic KAr modulating GABA release, KAr have also been found to contribute to the EPSCs in interneurones in CA1 (Cossart *et al*., 1998; Frerking *et al*., 1998). Recently, these KAr have been shown to be more effective than AMPAr in increasing interneurone firing frequency, probably as a result of their long decay times (Yang *et al*., 2007). Yang *et al*. (2007) also noted that single synaptic KAr mediated currents were capable of shifting the phase of interneurone firing, which they suggested could mean that KAr may play an important role in the synchronisation of interneurones in CA1. However, as yet, the hippocampus is the only region in which direct evidence has been produced for the presence of KAr acting to drive interneurones (Frerking *et al*., 1998; Yang *et al*., 2006b; 2007; Goldin *et al*., 2007).

At present, nothing is known about the role of KAr at inhibitory synapses in the EC. Thus, this chapter had two main aims: 1) to determine if presynaptic KAr were present in inhibitory synapses in layer III of the EC and 2) if KA heteroreceptors are present, how do they act to modulate GABA release.

5.2. Methods

Most methods were as described in Chapter 2. In all recordings of IPSCs, 2-AP5 (50 μM), GYKI 53665 (25 μM) or NBQX (1 μM), and CGP 55845 (5 μM) were included in the bath to block NMDAr, AMPAr and $GABA_B$r respectively. To investigate the function of KAr the GluR5 selective antagonist, UBP 302, and the GluR5 selective agonist, ATPA, were used.

5.3. Results

5.3.1. Effects of KAR ligands on sIPSCs

5.3.1.1. ATPA increases spontaneous activity

The effects ATPA (0.1 μM and 0.5 μM) on sIPSCs were assessed on 7 neurones. Addition of ATPA (0.1 μM) decreased IEI from 122 ± 14 ms to 98 ± 8 ms (KS test – $P<0.0001$, n=7), which reflects a increase in mean frequency from 7.3 ± 0.8 Hz to 10.7 ± 1.1 Hz (+ 53.1 ± 17.5 %). ATPA (0.5 μM) further decreased the IEI to 73 ± 12 ms (KS test – $P<0.0001$, n=7; Figure 5.1A,C), which reflects a mean frequency of 16.6 ± 3.6 Hz, and therefore an increase of 65.3 ± 40.0 %. Concurrently, there was no change in amplitude or kinetics (Table 5.1; Figure 5.1B).

Some studies have shown that higher concentrations of KAr agonist are capable of decreasing GABA release when lower concentrations facilitate release (Braga *et al*, 2003). Therefore I tested the effects of a higher concentration of ATPA on sIPSCs in a further 7 neurones. ATPA (10 μM) also caused a decrease in inter-event interval from 126 ± 25 ms in control to 72 ± 24 ms (KS test – $P<0.0001$, n=7; Figure 5.1A,C), which reflects a mean increase in frequency from 10.8 ± 3.5 Hz to 22.7 ± 5.3 Hz (+ 51.6 ± 14.2 %). There was no concurrent change in kinetics of the events (Table 5.1; Figure 5.1B). However, there was a tendency for mean amplitude to be increased in

the presence of this higher concentration of ATPA (Table 5.1). After the initial increase in frequency, prolonged exposure (> 15 minutes) to this higher concentration caused a subsequent decrease frequency, (16.7 ± 4.9 Hz; $P<0.01$, n=7).

sIPSCs	IEI (ms)	Amplitude (pA)	Decay Time (ms)	Rise Time (ms)
Control	122 ± 14	37.5 ± 2.4	4.8 ± 0.3	2.3 ± 0.1
+ ATPA (0.1 µM)	98 ± 8 *	35.4 ± 2.6	4.8 ± 0.3	2.3 ± 0.2
+ ATPA (0.5 µM)	73 ± 12 * †	34.8 ± 3.2	5.4 ± 0.4	2.7 ± 0.3
+ ATPA (10 µM)	72 ± 23 *	42.2 ± 6.1	4.7 ± 1.0	2.0 ± 0.5

Table *5.1. Effects of ATPA on IEI, amplitude and kinetics of sIPSCs.* * *indicates a significant change when compared control (*P *<0.001 KS Test).* † *indicates a significant change when compared to 0.5 µM ATPA (*P *<0.001 KS Test).*

5.3.1.2. Effects of ATPA on mIPSCs

The results of these experiments indicate a role for KAr in the control of GABA release in the EC. The increase in frequency of sIPSCs without change in amplitude or kinetics is indicative of presynaptic receptors on the terminals acting to increase quantal GABA release. However, these results cannot rule out the alternative possibility that there are KAr postsynaptic to glutamate terminals on the soma and/or dendrites acting to drive GABA release by exciting the interneurones, increasing firing, and thus resulting in increased activity-dependent release from the inhibitory terminals on the recorded neurones.

To further elucidate the location of these receptors I looked at the effect of ATPA (0.5 µM) on mIPSCs recorded in the presence of TTX (1 µM) which effectively blocks action potential-dependent release. Under these conditions ATPA (0.5 µM) still caused a decrease in IEI of mIPSCs from 293 ± 31 ms to 240 ± 27 ms (n = 10, $P<0.001$) reflecting an increase in mean frequency from 4.0 ± 0.6 Hz to 4.9 ± 0.8 Hz (+ 23.3 ± 4.0 %). Proportionally this change was not dramatically different to that seen with sIPSCs, but in terms of overall increase in frequency it appeared much less. Again there was no change in mean amplitude, decay time or 10-90 % rise time (Table 5.2).

mIPSCs	IEI (ms)	Amplitude (pA)	Decay Time (ms)	Rise Time (ms)
Control	293 ± 31	29.8 ± 2.0	5.2 ± 0.6	2.5 ± 0.4
+ ATPA (0.5 µM)	240 ± 27 *	31.3 ± 2.8	5.3 ± 0.7	2.3 ± 0.4

Table 5.2. Effects of ATPA on IEI, amplitude and kinetics of mIPSCs. * *indicates a significant change when compared control (*P *<0.001 KS Test).*

In 4 of these neurones, I tested the effects of UBP 302 (20 µM) on the increase in frequency mediated by ATPA. In these neurones, the mean IEI in control conditions was 359 ± 91 ms and this decreased to 262 ± 82 ms in the presence of ATPA (KS test – P<0.001; n=4). Subsequent addition of UBP 302 had no significant effect (270 ± 68 ms). This was unexpected as UBP 302 should be selective for GluR5 receptors and would be predicted to reverse the effects of the GluR5 agonist. Therefore, we repeated the experiments in a further 3 cells, but applied UBP 302 prior to ATPA. UBP 302 (20 µM) had no significant effect on IEI (from 286 ± 135 ms to 239 ± 101 ms). However, ATPA (0.5 µM) still caused a decrease in IEI to 94 ± 29 ms, reflecting a mean increase in frequency from 7.4 ± 4.2 to 12.6 ± 3.3 Hz (137.0 ± 62.8 %).

These results suggest that the KAr responsible for increasing GABA release are at least partly located on the GABA terminals. However, the effect of ATPA did appear to be proportionally less in the presence of TTX, so GluR5 receptors may also be present on the interneurone cell bodies or dendrites.

The failure of UBP 302 to block the increase in mIPSCs evoked by ATPA could suggest the agonist is acting via a subtype of GluR5 receptor not sensitive to UBP 302, or even via a receptor <u>not</u> containing the GluR5 subunit.

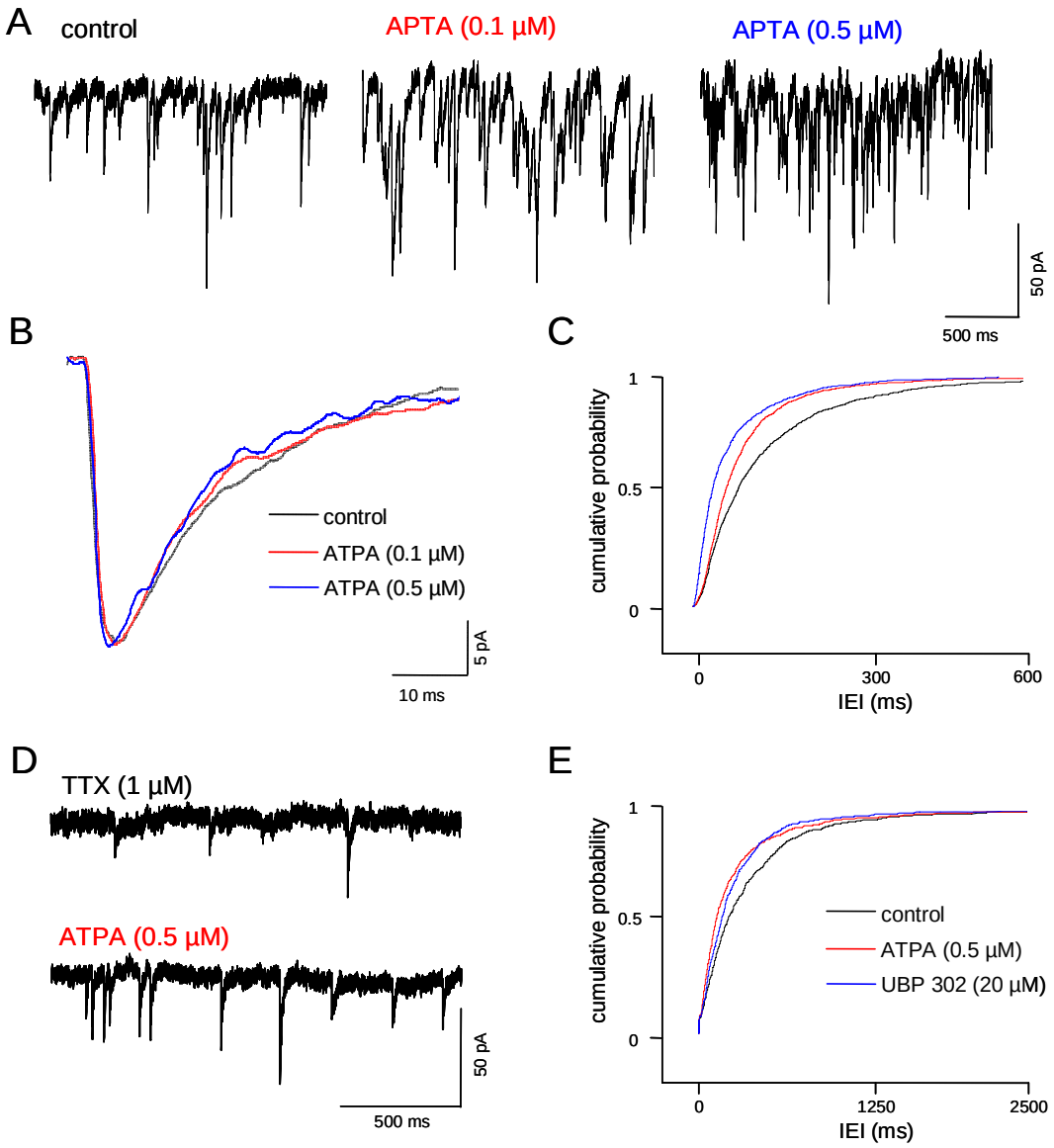


Figure *5.1. Effects of ATPA on sIPSCs and mIPSCs.* *A: Concurrent recordings showing sIPSCs in control conditions and in the presence of ATPA (0.5 µM and 10 µM) in a typical layer III neurone. B: Average IPSC (n=60) in control conditions and after addition of ATPA. C: Cumulative probability graph of sIPSC IEI control conditions and after ATPA application (n=6). D: Concurrent recordings showing mIPSCs in an example cell in control conditions and in the presence of ATPA. E: Cumulative probability graph of mIPSC IEI control conditions and after ATPA application (n=4).*

5.3.1.3. UBP 302 and CNQX reduce sIPSC frequency

In these experiments, as previously, sIPSCs were recorded in the presence of GYKI 53665 and 2-AP5 to block AMPAr and NMDAr respectively. In 6 neurones the control IEI was 86 ± 23 ms. Addition of UBP 302 increased IEI to 119 ± 22 (KS test $P<0.05$, n=6; Figure 5.2B) which reflects a decrease in frequency from 15.4 ± 3.2 Hz to 10.1 ± 2.1 Hz (- 31.3 ± 5.8 %). Subsequent addition of CNQX (10 µM) to the same

neurones caused a further significant increase in IEI to 154 ± 24 (KS test $P<0.0001$, n=6; Figure 5.2B) which reflects a decrease in frequency from 10.1 ± 2.1 Hz to 7.1 ± 0.9 Hz (20.11 ± 10.5 %). Neither antagonist had any effect on amplitude or kinetics (Table 5.3; Figure 4.2C). Unlike the sEPSC the train of evoked stimuli didn't cause a short-term increase in the number of sIPSCs. Thus, the combined effect of CNQX and UBP 302 suggest that there may be more than one KAr controlling GABA release. The effects of ATPA on sIPSCs and mIPSCs (Section 5.3.1.1 and 5.3.1.2.) suggest that GluR5 KAr are present on both GABA terminals and the soma/dendrites or the GABAergic interneurones. UBP 302 decreases sIPSC frequency, but not mIPSCs (Section 5.3.1.2). This indicates that there may be KAr mediating a tonic excitatory drive onto interneurones but that GluR5 on the terminal are unlikely to be tonically activated by ambient glutamate. CNQX may be blocking a KAr on the interneurones as well, but until experiments have been done on mIPSCs in TTX we cannot rule out that this receptor may also be tonically activated on the terminals as well.

sIPSCs	IEI (ms)	Amplitude (pA)	Decay Time (ms)	Rise Time (ms)
Control	86 ± 23	34.6 ± 2.5	8.9 ± 1.3	1.7 ± 0.2
+ UBP 302 (20 μM)	119 ± 22 *	32.1 ± 1.8	8.3 ± 1.4	1.6 ± 0.1
+ CNQX (10 μM)	154 ± 24 †	30.3 ± 2.2	9.0 ± 1.5	1.9 ± 0.2

Table 5.3. Effects of UBP 302 and CNQX on IEI, amplitude and kinetics of sIPSCs. ** indicates a significant change when compared to control (*P *<0.001 KS Test). † indicates a significant change when compared to UBP (*P *<0.001 KS Test).*

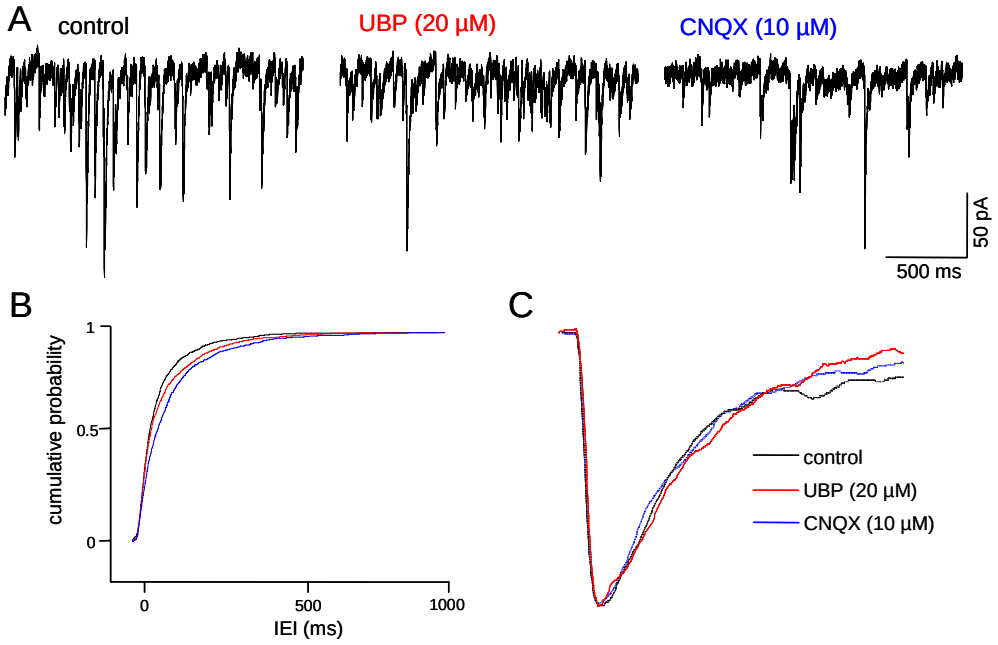


Figure 5.2. Effects of KAr antagonists on sIPSCs.. *A: Concurrent recordings of sIPSCs in control conditions and in the presence of UBP 302 (20 µM) and CNQX (10 µM) in a typical layer III neurone. B: Cumulative probability graph of IEI of sIPSCs in control conditions and after application of UBP 302 and CNQX (n=6). C: Average IPSC (n=60 in control conditions and after application of UBP 302 and CNQX*

5.3.2. Presynaptic KAr act via VGCC

5.3.2.1. Effect of Cd^{2+} on the facilitation of GABA release mediated by KAr

The preceding experiments on sIPSCs and mIPSCs suggested that the KAr that facilitate GABA release are located, both on the soma/dendrites of interneurones and on the GABAergic terminals. Activation of the receptors on the terminals could increase release by increasing presynaptic Ca^{2+} entry. KAr, depending on their subunit composition and post-translational editing (Burnashev *et al*., 1996), are permeable to calcium although they are normally more permeable to monovalent cations. Thus, Ca^{2+} entry through the receptors could increase GABA release. Alternatively, the receptors could depolarise the terminal and activate VGCC to enhance release. To try to determine which of these scenarios is most likely I looked at the effect of Cd^{2+}, a non-selective blocker of VGCC. I assessed the effect of the divalent cation on the increase in frequency of sIPSCs induced by ATPA. In 8 neurones, ATPA (0.5 µM) decreased IEI from 121 ± 33 ms to 75 ± 16 ms (KS test – P<0.0001; n=8), which represents a increase in mean frequency from 13.1 ± 2.9 Hz to 19.4 ± 4.4 Hz (+ 55.9 ± 14.8 %). Subsequent addition of Cd^{2+} (50 µM) partially reversed the decrease in IEI to

96 ± 20 ms (KS test – $P<0.001$; n=8) reflecting a mean frequency of 15.0 ± 3.4 (decrease of 21.7 ± 3.5 %). Therefore, Cd^{2+} partially blocks the increase in frequency of sIPSCs mediated by ATPA. This suggests that VGCC are important for the increase in GABA release elicited by GluR5 activation. However, this doesn't distinguish between action potential-dependent and independent release, as ATPA may also be activating KAr which are not located directly on the terminal. Thus, I repeated the experiments looking at mIPSCs. In these experiments I added Cd^{2+} before ATPA to ensure that it was having no direct effects on the mIPSCs. Indeed, Cd^{2+} had no effect on IEI, amplitude or kinetics of the mIPSCs (Table 5.4; Figure 5.3A,B). When ATPA (0.5 µM) was now applied in the presence of Cd^{2+} (50 µM) it failed to increase IEI (from 252 ± 29 ms to 251 ± 31 ms; n=9; Figure 5.3A,B). Thus, these experiments strongly suggest that the main facilitatory effect on GABA release of the KAr on the terminals, due to depolarisation, presumably as a result of Na^{+} entry, and subsequent opening of VGCC.

mIPSCs	IEI (ms)	Amplitude (pA)	Decay Time (ms)	Rise Time (ms)
Control	283 ± 32	30.3 ± 2.2	5.3 ± 2.1	2.5 ± 1.4
+ Cd^{2+} (50 µM)	252 ± 29	27.8 ± 3.2	4.5 ± 0.2	2.4 ± 0.7
+ ATPA (0.5 µM)	251 ± 31	27.4 ± 2.0	4.4 ± 0.2	2.3 ± 0.4

Table 5.4. Effects of ATPA and Cd^{2+} on IEI, Amplitude and kinetics of mIPSCs.

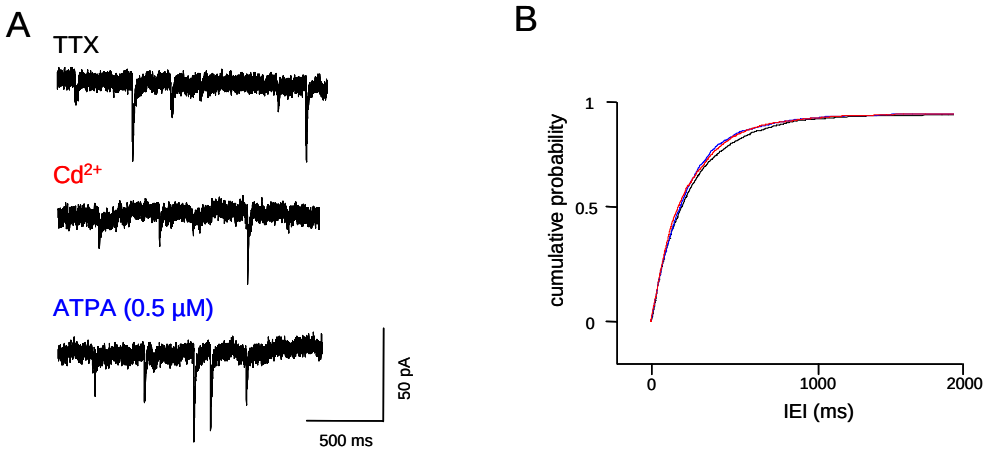


Figure 5.3. Effects of Cd^{2+} and APTA on mIPSCs. *A: Concurrent recordings of mIPSCs in control conditions and after Cd^{2+} (50 µM) and ATPA (0.5 µM) application in a typical layer III neurone. B: Cumulative probability graph of mISPC IEI in control conditions and after addition of Cd^{2+} and ATPA (n=9).*

5.3.3. Analysis of KAR ligands effects on eIPSCs

5.3.3.1. eIPSCs in layer III

Using a bipolar stimulating electrode positioned in layer V of the lateral EC a train of IPSCs can be evoked in neurones in layer III of the medial EC. In these experiments, AMPAr and NMDAr were again blocked, and the stimulating electrode was placed relatively close to the recording site. I evoked trains of 5 responses at 5 and 10 Hz. With both frequencies, the pattern and profile of responses was similar. There was a clear cut paired pulse depression (PPD), a decrease in the amplitude of the second response when compared to the first response in the train. Thereafter, the responses remained depressed throughout the train. At 5 Hz (equating to a paired pulse interval of 200 ms) the ratio of depression between the first and second response was 0.5 ± 0.1 (n=8), this was very similar to the depression seen at 10 Hz (0.5 ± 0.1; n=7). Average amplitudes and decay times for the five responses in the 5 and 10 Hz trains can be seen in Table 5.5. Graphs showing the normalised average peak amplitude for pooled data and paired pulse ratios can be seen in Figure 5.4. I tested the effects of a $GABA_B$ antagonist, CGP 55845A, on the profile of the 5 Hz train of eIPSCs. There was no significant effect of the antagonist on either PPR (1.6 ± 0.1; n=3) or decay times of any of the responses in the train (Table 5.5), therefore in future experiments data with and without CGP 55845A (5 µM) in the aCSF was pooled. eIPSCs were abolished by bicuculline (5 µM), indicating they are mediated by $GABA_A$r (Figure 5.4A).

		1	2	3	4	5
5 Hz	Amplitude	96.9 ± 13.1	49.7 ± 7.2	59.0 ± 10.7	65.9 ± 15.7	60.2 ± 1.0
	Decay time	10.9 ± 1.4	9.8 ± 1.4	8.2 ± 1.1	8.4 ± 1.0	7.7 ± 1.4
5 Hz + CGP 55845A	Amplitude	177.0 ± 21.0	98.5 ± 12.9	73.5 ± 4.5	120.9 ± 6.4	149.1 ± 42.7
	Decay time	11.9 ± 0.3	8.8 ± 1.7	8.9 ± 1.4	10.0 ± 1.5	9.6 ± 2.2
10 Hz	Amplitude	128.6 ± 40.8	59.6 ± 9.4	62.0 ± 10.5	73.6 ± 14.4	61.2 ± 11.3
	Decay time	9.7 ± 1.0	11.8 ± 1.4	9.4 ± 1.3	10.0 ± 1.7	9.1 ± 1.2

Table 5.5. Average amplitudes (pA) and decay times (ms) for eIPSCs.

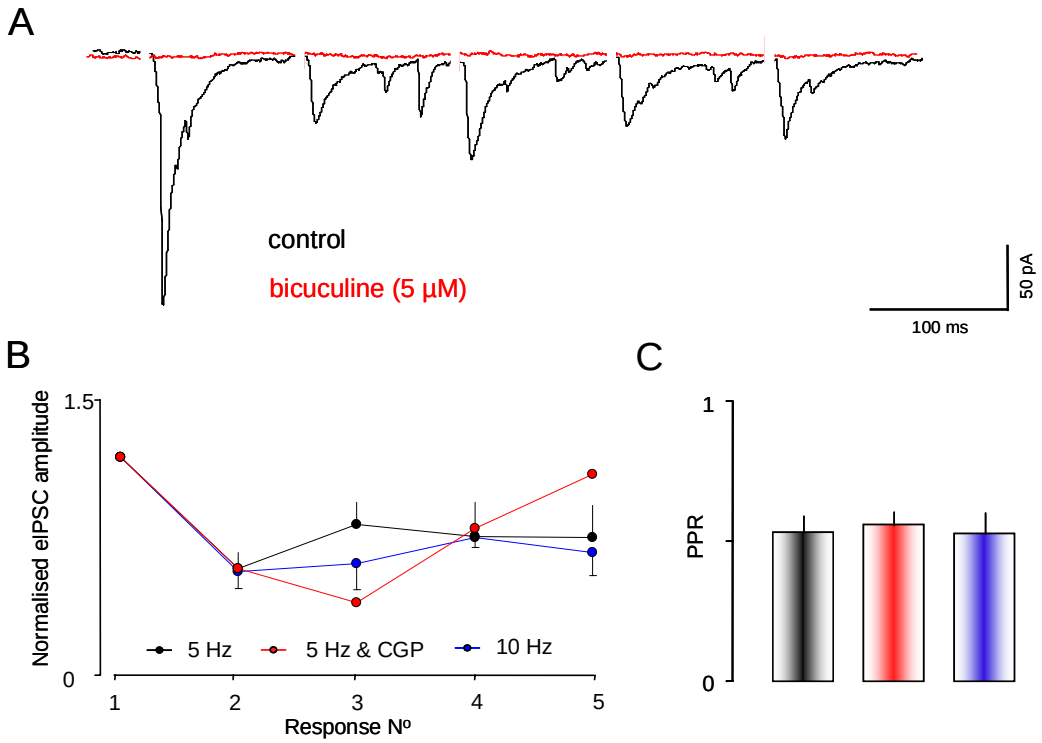


Figure 5.4. Evoked IPSCs in layer III of the EC. *A: Average trace of eIPSCs to trains of 5 shocks at 10 Hz recorded in a typical layer III neurone in control conditions and after addition of bicuculline (5 µM). B: Graph of normalised amplitudes of responses to trains of 5 shocks for 5 and 10 Hz and also 5 Hz in the presence of CGP 33845A (5 µM). C: Graph of PPR at different frequencies.*

5.3.3.2. ATPA increases amplitude of eIPSCs

Results from experiments on spontaneous release suggest that KAr facilitate GABA release via receptors on terminals and the interneurone soma and/or dendrites. However, the pharmacology of these effects is not clear cut. To look further at the role of KAr in control of inhibition I examined eIPSCs recorded as above. The effects of ATPA (0.1 and 0.5 µM) on eIPSCs (5 pulses at 5Hz; Figure 5.3A) were assessed in 8 cells. In these cells a concentration-dependent increase in the amplitude of all events across the train was seen in the presence of ATPA. After the addition of ATPA (0.1 µM) the average eIPSC amplitude of the first response was increased from 163.7 ± 10.2 to 204.2 ± 13.6 pA, (P<0.05, n=8). Likewise, the subsequent events in the train were also increased (P values ranging from <0.05 to 0.01), although the depression between the first and second responses and the overall train profile were largely unaltered (Figure 5.5A). When a higher concentration of ATPA was used there was little further increase in the first response (210.2 ± 13.1 pA; n=8). However there was a significant increase in the size of the second response (from 135.98 ± 10.71 pA to

186.33 ± 17.87 pA; *P*< 0.05; n=7). The subsequent responses in the train were also generally increased, such that all responses were approximately the same amplitude as the first, although these increases were not found to be significant when compared to the amplitudes in the lower concentration. The PPR increased in the presence of 0.5 µM ATPA (from 0.66 ± 0.03 to 0.89 ± 0.08; *P*<0.05, n=8; Figure 5.5B) reflecting the relative overall increase in the amplitude of the second response. Thus, with lower concentrations of ATPA, the depressive profile of the repetitive trains was maintained, whereas the higher concentrations essentially eliminated frequency-dependent depression. ATPA had no effect on decay time or 10-90% rise time of any of the evoked responses (Table 5.6).

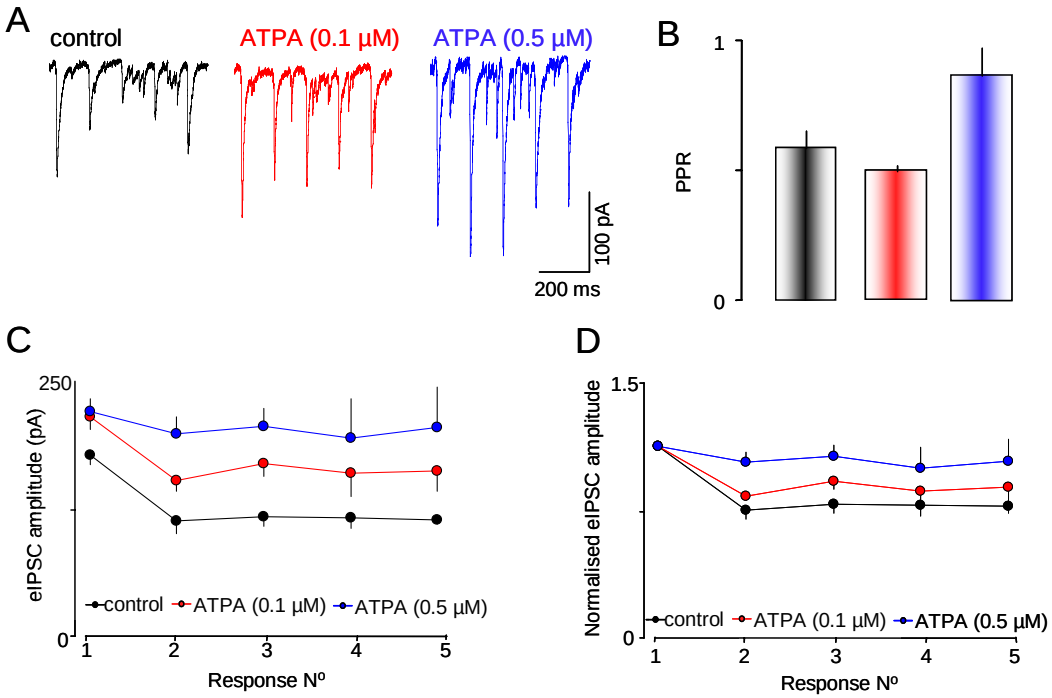


Figure 5.5. Effects of ATPA on eIPSCs. *A: Consecutive eIPSCs during 10 Hz train in control conditions and during perfusion of ATPA (0.1 and 0.5 µM) in a typical layer III neurone. B: Graph of PPR in control conditions and in the presence of ATPA. C: eIPSC mean amplitude graph in control conditions and after ATPA application (n=10) D: Graph of normalised amplitude of eIPSCs in control conditions and after ATPA application (n=10).*

5.3.3.3. UBP 302 and CNQX have different effects on eIPSCs

The previous results suggest that KAr can enhance GABA release at terminals in layer III. I have now looked at the effects of KAr antagonists to determine if these receptors

could be activated during repetitive stimulation with AMPAr and NMDAr blocked, and thus alter evoked GABA release.

The effects of UBP 302 (20 µM) on eIPSCs (5 pulses at 5 Hz; Figure 5.6A) were assessed in 6 neurones. As described above, in all cells there was a large decrease in amplitude of the second, and all subsequent responses in the train compared to the first. The average amplitude decreased from 163.5 ± 20.7 pA to 94.2 ± 18.8 pA (P<0.01, n=6) from the first to the second pulse. After the addition of UBP 302 the amplitude of first response was significantly decreased to 88.4 ± 7.4 pA (P<0.01; n=6). However, the amplitude of the second response increased to 121.8 ± 12.6 pA (Figure 5.1B). The change in the paired pulse ratio was highly significant (0.6 ± 0.1 to 1.4 ± 0.2, P<0.01, n=6; Figure 5.1C). Although, subsequent responses declined slightly in amplitude, they remained above the amplitude of the first response. The change in profile of the responses meant that the original PPD was converted into a substantial PPF by the GluR5 antagonist. Because UBP 302 reduced the first response in the train, this would suggest that Glur5 receptors may be activated in the interneurones soma/dendrites by the stimulation causing excitation of glutamatergic afferents to the interneurones. Blockade of this KAr drive would then lead to a decrease in GABA release onto the recorded neurone.

In all neurones, CNQX (10 µM) was then added. CNQX had no further effect on the amplitude of the first response (93.1 ± 11.9 pA). Now, however, the second and third responses remained approximately the same amplitude as the first (91.9 ± 9.1 pA; P<0.01, n=6). This meant that the paired pulse ratio (1.0 ± 0.1; P<0.05, n=6) was significantly different to both control and UBP 302 and was essentially at unity, reflecting the lack of frequency dependence. Neither UBP 302 (15 µM) nor CNQX (10 µM) had any effect on the decay times of any of the evoked responses (Table 5.6).

This would suggest again that two different KAr may be involved in modulating GABA release. The lack of effect on the first response indicates that the CNQX sensitive receptors are <u>not</u> involved in the excitatory input to the interneurones. The block of PPF may suggest that it is located on the terminals. This is speculated on further below.

		1	2	3	4	5
control	Amplitude	163.7 ± 10.2	92.9 ± 13.3	97.7 ± 10.7	96.4 ±11.3	94.6 ± 5.4
	Decay time	11.4 ± 2.3	10.0 ± 2.2	10.7 ± 2.5	10.7 ± 2.6	9.9 ± 1.9
+ ATPA	Amplitude	204.2 ± 13.6*	136.0 ± 13.6**	154.0 ± 13.3^**	144.2 ± 25.2*	146.6 ± 21.1*
	Decay time	10.6 ± 1.8	11.1 ± 2.1	10.8 ± 1.8	11.2 ± 1.4	10.7±2.0
+ UBP 302	Amplitude	88.4 ± 7.4*	121.8 ± 12.6	102.9 ± 12.0	106.7 ± 16.5	98.2 ± 11.9
	Decay time	12.6 ± 3.0	11.7 ± 2.2	11.0 ± 2.3	11.2 ± 3.0	9.1 ± 2.4
+ CNQX	Amplitude	93.1 ± 11.9*	91.9 ± 9.1†	87.7 ± 6.9*†	71.9 ± 5.5*	86.8 ± 15.1*
	Decay time	10.2 ± 2.3	11.9 ± 2.6	13.5 ± 3.2	11.7 ± 2.2	10.7 ± 2.1

Table 5.6. Amplitudes (pA) and Decay times (ms) for eIPSCs. ** indicates a significant change when compared to control (*P<0.05 **P<0.01; One way ANOVA and Bonferroni Test). † indicates a significant change when compared to UBP 302 (P < 0.01; One way ANOVA and Bonferroni Test).*

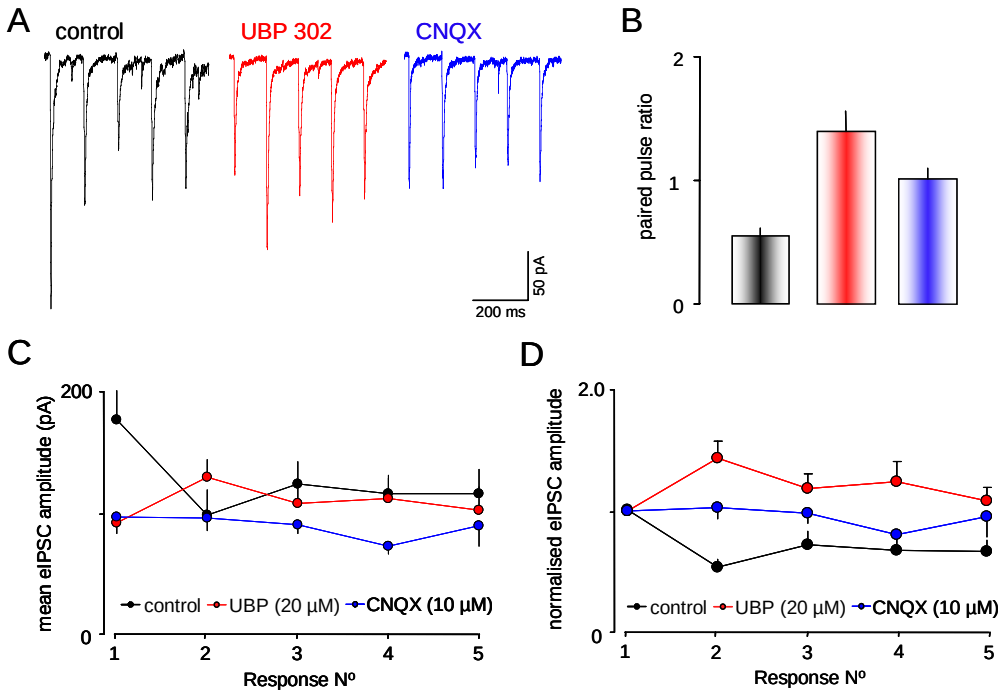


Figure 5.6. Effects of UBP 302 and CNQX on eIPSCs. *A: Consecutive eIPSCs during 10 Hz train in control conditions and during perfusion of UBP 302 (20 µM) and CNQX (10 µM). B: Graph of PPR in control conditions and in the presence of UBP 302 and CNQX. C: Graph of mean amplitude of eIPSCs in control conditions and after UBP 302 and CNQX application (n=10) D: Graph of normalised amplitude of eIPSCs in control conditions and after UBP 302 and CNQX application (n=10).*

5.4. Discussion

GABAergic transmission in the EC is modulated by a number of different receptors including NMDAr, mGluR and $GABA_Br$ (Jones and Woodhall, 2005). In other brain regions, particularly the hippocampus, presynaptic KAr have proven to be very important in the regulation of GABA release (Cossart *et al*., 2001; Frerking *et al*., 2001; Min *et al*., 1999; Rodriguez-Moreno *et al*., 2000). The aim of this study was to determine whether KAr are present on interneurones in layer III of the EC and, if so, what role they play in GABAergic transmission.

ATPA increased sIPSC frequency in layer III, and this facilitation partly persists in the presence of TTX. This, together with the lack of effect of the agonist on amplitude and kinetics of sIPSCs or mIPSCs strongly indicates that KAr act to facilitate spontaneous GABA release from interneurones in layer III of the EC. The effect of ATPA was reduced, but not abolished, by TTX, suggesting that KAr are active at two sites: 1. on the soma or dendrites of the interneurones where their activation could increase neuronal firing and hence activity-driven GABA release. 2. On the GABAergic terminals, where they could directly enhance glutamate release. As ATPA shows good selectivity for receptors containing the GluR5 subunit (Hoo *et al*., 1999; Stensbøl *et al*., 2001; Nielsen *et al*., 2003), this could suggest that these are the receptors most likely to be involved at both sites. However, recent evidence suggests that ATPA has some activity at GluR6 receptors when they also contain the KA2 subunit (Paternain *et al*., 2000). Both of these subunits are also present in the EC (Bahn *et al*., 1994). Thus, it is possible that ATPA could be activating different receptors at the two sites. Although, the increase in sIPSC frequency elicited by ATPA was partially reduced by a GluR5 antagonist, and the increase in frequency of mIPSCs caused by ATPA was not blocked by UBP 302 (the GluR5 selective antagonist). This may indicate the presence of presynaptic KAr on the terminals acting to facilitate GABA release and containing the GluR6/KA2 subunits, and that there are also somatic KAr driving the interneurones, which contain the GluR5 subunit. It's also interesting there is no increase in events directly after a 5 Hz stimulus train, as seen for EPSCs. The results of studies with eIPSCs generally support the conclusion from studies on sIPSCs that there are excitatory GluR5 KAr on the interneurone soma/dendrites and a different, possibly GluR6/KA, receptor on the terminals. These are discussed further below.

These results are unlike what has been found in the hippocampus, in that presynaptic KAr in CA1 act to depress GABA release. Rodriguez *et al*. (1997) showed that application of kainate reduced the frequency of mIPSCs and the amplitude of eIPSCs, in culture and hippocampal slices. The KAr responsible for this depression are thought to contain the GluR5 subunit, as ATPA has been shown to cause a reduction in eIPSC amplitude which can be reversed by application of the GluR5 selective antagonist, LY 294486 (Clarke *et al*., 1997). KAr have been found to contribute to the EPSCs in interneurones in the hippocampus (Cossart *et al*., 1998; Frerking *et al*., 1998), similar to what I have proposed may be the case in the EC. KAr have are thought to be more effective than AMPAr in increasing interneurone firing frequency, due to their long decay times (Yang *et al*., 2007). These KAr present on the soma/dendrites of hippocampal interneurones are also thought to contain the GluR5 subunit, as they can be activated by ATPA and blocked by the GluR5 antagonist, LY 293558 (Cossart *et al*., 1998).

Presynaptic KAr have been shown to bidirectionally modulate both glutamate release (Schmitz *et al.,* 2000; 2001; Lauri *et al.,* 2006) and GABA release (Braga *et al.,* 2003; Jiang *et al*., 2001). This intriguing ability allows them to facilitate transmitter release at low concentrations, but depress release at higher concentrations at the same synapse. It appears unlikely that this phenomenon occurs for GABA release in layer III of the EC, as both low and higher concentrations of ATPA (10μM) only caused an increase in sIPSC frequency. In the amygdala the same high concentration causes a decrease in the frequency of mIPSCs and increases the number of synaptic failures. I did see a decrease in the frequency of spontaneous events after prolonged exposure to the higher concentrations of ATPA, however, this was preceded by a pronounced increase in frequency. It could be that the increase in frequency resulted from low concentrations of ATPA as it washed into the bath and that the decrease was the result of the high concentration, when it had reached equilibrium in the slices. A biphasic effect similar to this was seen in the hippocampus (Jiang *et al*., 2001) where success rate of unitary IPSCs increased for the first few minutes of KA application before decreasing after 3-5 minutes. However, unlike in the hippocampus, in my experiments the increase in sIPSCs often persisted for up to 15 minutes, when equilibrium would have already been attained. Also, not all cells displayed a decrease. An alternative

explanation is that the decrease was due to a depletion of the GABA stores in these terminals and not a direct effect of the activation of KAr.

I have attempted to determine the mechanism by which KAr facilitate GABA release. In my studies, Cd^{2+} completely prevented the increase in frequency of mIPSCs caused by ATPA. This suggests that VGCC are involved in the mechanism of GABA facilitation in these synapses. A likely scenario is that the KAr are acting to depolarise the terminal, thus activating VGCC and causing a subsequent influx of calcium and enhancement of release. Other studies have also looked at the mechanisms of KAr effects on GABA release (Cossart *et al*, 2001., Braga *et al*., 2003; Mathew and Hablitz, 2008). The effects of Cd^{2+} on facilitation of release have been investigated in the hippocampus and in the amygdala, where KA/ATPA-induced increase of the frequency of mIPSCs in the presence of Cd^{2+} was not significantly different from that in the absence of Cd^{2+} (Cossart *et al*., 2001; Braga et al., 2003; Mathew and Hablitz, 2008). Therefore, it seemed unlikely that VGCC were involved in these brain regions. In addition, further studies have suggested that extracellular Ca^{2+} was necessary, as exposure to solutions containing no added extracellular Ca^{2+} inhibited the KAr-mediated facilitation. In addition, KA was unable to induce facilitation in the presence of an inhibitor of Ca^{2+} permeable AMPA/KAr, suggesting that Ca^{2+} entry via Ca^{2+}-permeable KAr is involved (Mathew and Hablitz, 2008). Facilitation was also blocked after inhibition of endosomal Ca^{2+}-ATPase-mediated Ca^{2+}uptake, suggesting a role for intracellular stores. Inhibition of IP_3 receptor, but not ryanodine receptor, induced Ca^{2+} release blocked KAr-induced changes, therefore the authors concluded that Ca^{2+} entering through Ca^{2+}-permeable presynaptic KAr results in Ca^{2+} release from intracellular stores through IP_3 receptors, which causes an increase in GABA transmission (Mathew and Hablitz, 2008). I cannot rule out the possibility that the KAr is acting via a metabotropic pathway in layer III, as has been suggested previously for the down-regulation of GABA (Rodriguez-Moreno and Lerma, 1998) and the facilitation of glutamate (Rodriguez-Moreno and Sihra, 2004) in the hippocampus and the bidirectional modulation of GABA in the amygdala (Braga *et al*, 2003), particularly as the GluR5 subunit appears to be able to activate a G-protein, which could modulate VGCC (Rozas *et al*., 2003). This will require further investigation.

The results with eIPSCs are somewhat more complicated than those for the eEPSCs in Chapter 4. Evoked sIPSCs in control conditions showed a clear PPD of approximately 50 %, a common feature at central GABAergic synapses. This has often been attributed to activation of presynaptic $GABA_B$ receptors (Davies *et al*., 1990; Deisz and Prince, 1989; Mott *et al*. 1993; Nathan and Lambert, 1991). However, $GABA_B$ blockade had little effect on PPD in my studies in layer III. Other mechanisms suggested to be involved include depletion of transmitter in the presynaptic terminal (Stevens and Tsujimoto, 1995; Debanne *et al*., 1996; Stevens and Wesseling, 1998), decreased calcium influx in response to action potential invasion (Brody and Yue, 2000), inactivation of presynaptic calcium channel (Patil *et al*., 1998) and post pulse hyperpolarisation due to activation of calcium activated potassium and/or chloride channels (Marrion and Tavalin, 1998). In principle, any of these could be involved in PPD seen in these cells in layer III but this requires further experimentation to elucidate the mechanism involved. What is very interesting is the modification of frequency dependence of GABA transmission by KAr at EC synapses. UBP 302 caused a depression in the amplitude of the first response to stimulation. By itself this would result in a reduction in PPD. However, UBP 302 also appeared to cause an increase in the second and subsequent events in the train, thus causing a shift in the PPR to facilitation. What are the mechanisms involved in this effect? A number of factors could account for the effect of UBP 302 on the amplitude of the initial response. It could be due to blockade of presynaptic GluR5 KAr on GABA terminals. In this scenario, the assumption would be that the first shock activated GABA inputs and simultaneously activated a parallel glutamate pathway. Glutamate released from this pathway would spillover to the GABA terminals, activating GluR5 receptors and instantaneously enhancing GABA release. Blocking these receptors would cause a decrease in the eIPSC to the first shock. The second possibility is that the GluR5 KAr are located on the interneurone soma/dendrites. Here, the first shock would activate both the GABA interneurones directly, but also feedforward glutamate pathways onto the interneurone. The amplitude of the first IPSC would depend on both pathways. UBP 302 would block the drive on to the interneurone and thus reduce the amplitude of the response. As earlier experiments showed that UBP 302 reduced sIPSC but not mIPSC frequency, and ATPA has a greater effect on sIPSCs than mIPSCs, this data seems to agree with the second proposal, and that there are GluR5 KAr driving excitation in interneurones in layer III. To determine whether or not there are postsynaptic GluR5 KAr located on the interneurones themselves, as has been found

in the hippocampus (Cossart *et al*., 1998; Frerking *et al*., 1998), we would need to record from the interneurones directly. These experiments should form part of future investigations.

If GluR5 receptor blockade reduces the amplitude of the first response, what is responsible for the switch from PPD to PPF? This could arise because less GABA is released during the first event, leaving more in a readily available pool for the second and subsequent events. However, addition of CNQX on top of UBP 302 prevented the facilitation without affecting the amplitude of the first eIPSC. This could indicate the involvement of a second KAr acting as in the first scenario above. Glutamate could be spilling over from excitatory synapses and activating a UBP 302-insensitive, CNQX-sensitive receptor on the GABA terminal. The change in eIPSCs induced by ATPA support this picture. Low concentrations of ATPA caused an increase in the amplitude of all events in the train (including the first), without affecting PPD. A higher concentration of ATPA increased the later events to a greater extent, resulting in elimination of PPD or PPF. As mentioned earlier, ATPA can act at GluR5 containing KAr as well as at GluR6/KA2 KAr (Paternain *et al*., 2000). These effects could reflect a differential, concentration-dependent increase in activation of somatic GluR5 and terminal GluR6/KA2r (see Figure 5.7). Overall, the data from studies of sIPSCs, mIPSCs and eIPSCs could support this spatial segregation of facilitatory KAr on GABA interneurones. However, it would perhaps be prudent to investigate the effects of mGluR ligands on the PPD and subsequent shift to PPF seen in these experiments. As group III mGluR have been found to depress spontaneous inhibition in layer V of the EC (Woodhall et al, 2001b), it would be necessary to rule out the possible involvement of mGluR in these observations.

One of the elements motivating the investigation of KAr in the EC is the possible therapeutic potential of KAr agonists/antagonists in TLE. KA itself has been long linked to epilepsy because KA treatment is a common method to induce animal models of TLE, with systemic or local application of KA inducing seizures and neuropathological changes closely reminiscent of those in TLE patients (Ben-Ari, 1985). This, in itself, suggests a possible role for KA antagonists as anti-convulsants, and is supported by evidence that KAr activation causes an overall disinhibition of the hippocampus (Rodriguez-Moreno *et al*., 1997). More recent research has provided direct evidence for the importance of GluR5 containing KAr. It has been shown that a

GluR5 selective antagonist, LY382884, protects against pilocarpine induced limbic seizures (Smolders *et al*., 2002). Also topiramate blocks EPSCs mediated by GluR5 KAr (Gryder and Rogawski, 2003) and selectively protect against seizures induced by ATPA (Kaminski *et al*., 2004). It is difficult to resolve how the facilitation of GABA release by KAr seen in this study may relate to this evidence for the benefits of KAr antagonists in epilepsy since GluR5 blockade would be expected to reduce GABA release and exacerbate epileptic activity. It is also worth noting that the old view, that epilepsy results from impaired inhibition and that anti-convulsants simply enhance or restore inhibition, is no longer tenable. GABAergic interneurones are now increasingly seen to be drivers and initiators of network synchrony (Cobb *et al*., 1995; Traub *et al*., 2004; Klausberger *et al*., 2005). In addition, pathological changes in interneuronal synchronisation of principle neurones (Macloczky and Freund, 2005; Cossart *et al*., 2005) could be involved in TLE. Thus, alteration of the KAr-mediated facilitation of GABA release could very well be an important therapeutic target in epilepsy. However, a perhaps more relevant study showed that ATPA can have an anti-epileptic effect, stopping the spread of seizures, and this was believed to be due to the increase in frequency of sIPSCs (Khalilov *et al*., 2002).

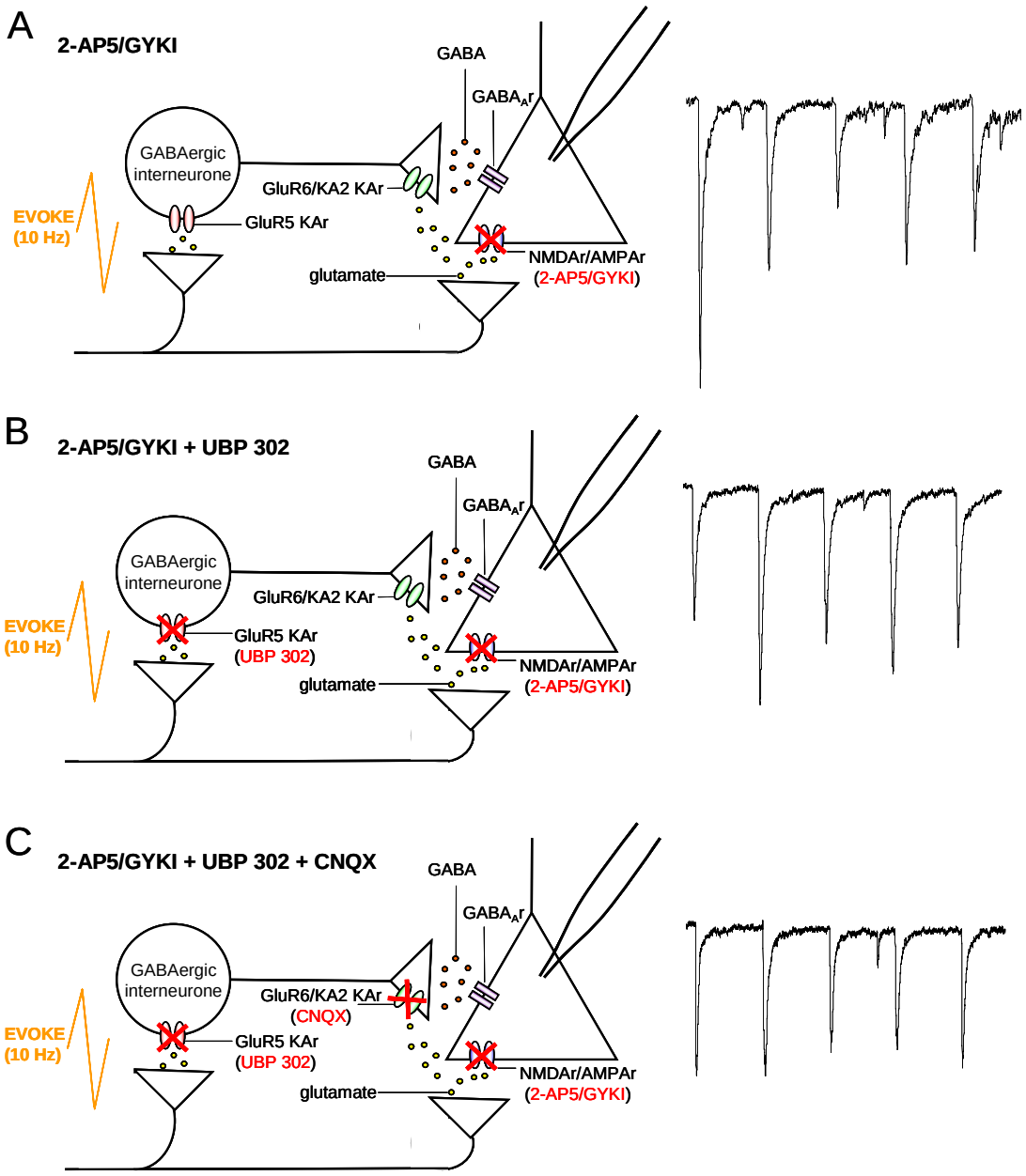


Figure 5.7. Schematic summary diagram of roles of KAr in control of GABAergic transmission. *Schematic diagrams showing what may be happening during 10 Hz stimulation in the presence of A: 2-AP5 (50 µM) and GYKI 53665 (25 µM) and B: after application of UBP 302 (20 µM) and C: CNQX (10 µM).*

Finally, related to this is the role of KAr in oscillations discussed in Chapter 1. It is clear that GluR5 KAr are involved in initiation and/or maintenance of both gamma oscillations and SWO (Cunningham *et al.*, 2003; Stranger *et al.*, 2008; Cunningham *et al.*, 2006). During SWO, both excitatory and inhibitory transmission appear to be increased (Sanchez-Vives and McCormick, 2000; Shu *et al.*, 2003). Thus, activation of KAr could be driving increased network synchrony by simultaneously elevating GABAergic and glutamatergic transmission and the interactions between them.

Whether or not KAr prove to be a new therapeutic target for epilepsy, it is clear that they play an important role in the modulation of GABAergic transmission layer III of the EC.

CHAPTER 6

EFFECTS OF REDUCING $[Mg^{2+}]_o$ ON EXCITATION AND INHIBITION IN LAYER III

6.1. Introduction

In Chapter 1, I described recent work by Cunningham *et al.* (2006b) that has indicated that KAr containing the GluR5 subunit are involved in the initiation and/or maintenance of SWO in the EC. In the previous data chapters I have now shown that GluR5 containing KAr are present in the EC. These are involved at both excitatory and inhibitory synapses acting to enhance release of glutamate and GABA. In addition, KAr also contribute to postsynaptic excitation at glutamate synapses. Indirect evidence also indicates that postsynaptic GluR5 KAr mediate, at least in part, an excitatory input to GABAergic interneurones.

SWO can be generated in the EC in response to reducing $[Mg^{2+}]_o$ from 2 mM to 1.25 mM (Cunningham *et al.*, 2006b; Greenhill and Jones, unpublished observations). Most researchers are familiar with the ability of Mg^{2+} to block most cation channels, particularly Ca^{2+} channels. Mg^{2+} is often referred to as a physiological calcium channel blocker, as small decreases in Mg^{2+} plasma levels can lead to a significant increase in evoked Ca^{2+} currents and in resting Ca^{2+} (Altura and Altura, 1995). Thus, the effect of reducing $[Mg^{2+}]_o$ would be to increase activity at VGCC and potentially increase the release of both glutamate and GABA.

A second, very important ion channel blocking activity of Mg^{2+} in the CNS is at the NMDAr, where the cation exerts a voltage-dependent block of the receptor ionophore. It has been accepted that neurones need to be depolarised to remove the Mg^{2+} block before the NMDAr can be activated by glutamate (Mayer *et al.*, 1984). This role of Mg^{2+} in the function of NMDAr has long been thought to be associated with the underlying mechanism for LTP and therefore vital for learning and memory (for review see Brown *et al.*, 1988; Collingridge and Bliss, 1995; Bennett, 2000). Thus, lowering $[Mg^{2+}]_o$ should lead to enhanced activation of NMDAr and this could also be involved in generation of SWO. It is well established that more profound reductions in $[Mg^{2+}]_o$ lead to highly synchronised and prolonged epileptiform activity in the EC, which is dependant on NMDAr activation (Walter *et al.*, 1986; Jones and Heinemann, 1988; Jones and Lambert, 1990a,b). Although the experiments of Cunningham *et al.* (2006b) reported that SWO were <u>not</u> altered by NMDAr antagonists, further studies in this laboratory (Greenhill and Jones, unpublished observations) have shown that SWO

induced by lowering $[Mg^{2+}]_o$ from 2.0 to 1.25 mM, can be reduced by 2-AP5 (see Figure 6.1 and below).

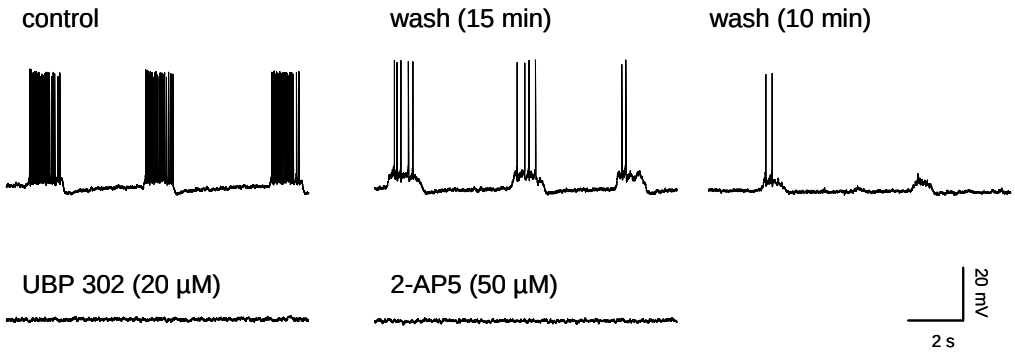


Figure 6.1. Effects of UBP 302 and 2-AP5 on SWO. *Consecutive intracellular recordings of SWO induced in the EC by reducing $[Mg^{2+}]_o$ showing the effects of UBP 302 (20 µM) and 2-AP5 (50 µM) and after washout (Greenhill and Jones, unpublished).*

Rhythmic electrical activity or oscillations of various frequencies are seen in many areas of the CNS and are important in many different processes, including memory (for review see Axmacher *et al.*, 2006). SWO, in particular, have been suggested to underlie the transfer of memory traces from the hippocampus to the neocortex during sleep (for review see Hoffman *et al.*, 2007), partly due to their prominence during slow wave sleep. As previously mentioned, KAr are thought to play a role in SWO generation in the EC, as these can be blocked by UBP 302 (Cunningham *et al.*, 2006b; Figure 6.2A). sEPSPs with noticeably long decay times are present in intracellular recordings just before and during the onset of the SWO (Cunningham *et al.*, 200b6; Figure 6.2B). These EPSCs have a significant KAr component as UBP 302 reduces the decay times of the events (Figure 6.1C), indicating the importance of the GluR5 containing KAr in SWO. As mentioned above, NMDAr may also be important in the generation of SWO. Experiments in this laboratory have shown the SWO in the EC can be blocked by UBP 302, but that they can also be reduced or blocked by 2-AP5 in the same neurone. Thus, KAr are involved although it is not a straightforward story. KAr have also been shown to play a role in other oscillations including gamma oscillations in the hippocampus (Brown *et al.*, 2006) and the EC (Cunningham *et al.*, 2003), theta oscillations in the hippocampus (Huxter *et al.*, 2007) and beta oscillations in the motor cortex (Yamawaki *et al.*, 2008).

In this chapter I have attempted to begin a clarification of the role of KAr in the generation of SWO by studying how circumstances that generate SWO may alter KAr

transmission. The work has had two main aims: 1) to investigate the effects of reducing Mg^{2+} on synaptic transmission at both glutamatergic and GABAergic synapses in the EC and 2) to identify how these changes are linked to KAr and their role in SWO.

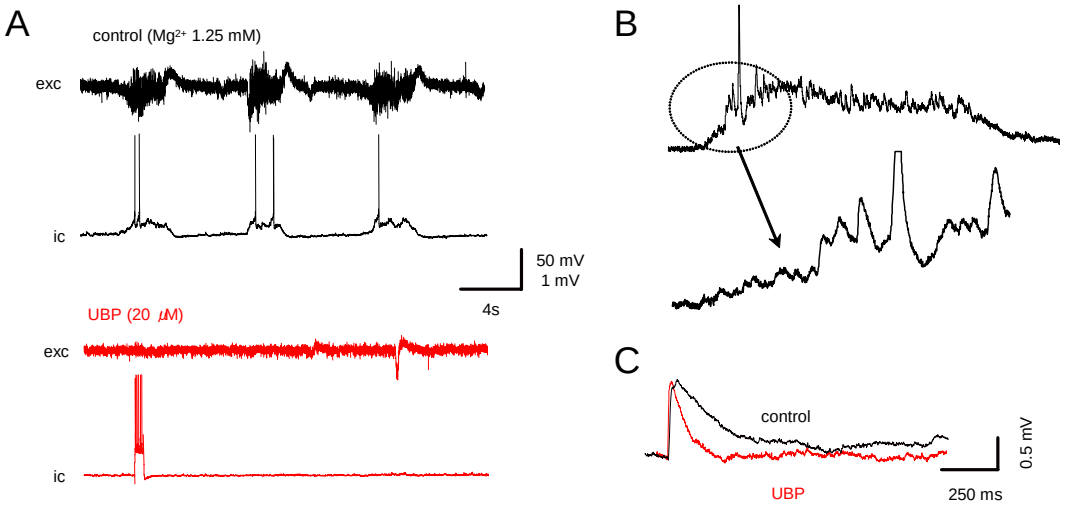


Figure 6.2. SWO in the EC. *A: Extra- (exc) and intracellular (ic) recordings of spontaneous SWO induced by lowering* $[Mg^{2+}]_o$ *(from 2 to 1.25 mM) in control conditions and after addition of UBP 302. B. Expansion of the ic record shows a flurry of spontaneous EPSPs during initiation of the SWO. C. Individual sEPSPs in control conditions and after addition of UBP 302. (adapted from Cunningham et al., 2006b).*

6.2. Methods

As in previous Chapters, brain slices were prepared from juvenile animals aged from 4 to 6 weeks old (45 - 70 g) and general methods are the same (Chapter 2). The normal aCSF employed contained 3 mM KCl and 2 mM $MgSO_4$. In the experiments where reduced $[Mg^{2+}]_o$ was required, the storage and recording aCSF was modified with slightly higher KCl (3.25 mM) and lowered $MgSO_4$ (1.25).

6.3. Results

6.3.1. Effects of reducing $[Mg^{2+}]_o$ on glutamatergic transmission.

6.3.1.1. Effect on sEPSCs.

In the first set of experiments I determined whether spontaneous glutamate release was altered by reducing $[Mg^{2+}]_o$. In 5 neurones, reducing $[Mg^{2+}]_o$ from 2 mM to 1.25

mM resulted in a substantial decrease in IEI from 180 ± 31 ms to 89 ± 16 ms (KS test – P<0.0001; n=5; see Figure 6.3). This reflects an increase in frequency from 6.2 ± 1.0 Hz to 12.7 ± 2.1 Hz. Analysis of the kinetics of the sEPSCs showed that there were no significant changes in the rise or decay times, although both tended to be faster in low Mg^{2+}. In addition, amplitudes were slightly, but not significantly smaller. All IEI, amplitudes and kinetics are recorded in Table 6.1. Thus, the general picture was an increase in frequency of events with little change in amplitude or kinetics. These results strongly suggest an overall increase in release without increased postsynaptic receptor activation. In particular, there was no suggestion for an increase in duration of events as a result of increased postsynaptic NMDAr or KAr activation.

sEPSC	IEI (ms)	Amplitude (pA)	Decay time (ms)	Rise Time (ms)
2.0 mM Mg^{2+}	180 ± 28	14.3±0.5	11.3±0.5	1.9±0.1
1.25 mM Mg^{2+}	89 ±15 *	12.8±1.2	8.3±0.6	1.4±0.2

Table 6.1. IEI, Amplitude and kinetic data for sEPSCs in 'normal' and reduced $[Mg^{2+}]_o$. ** indicates a significant change when compared control (P <0.001 KS Test).*

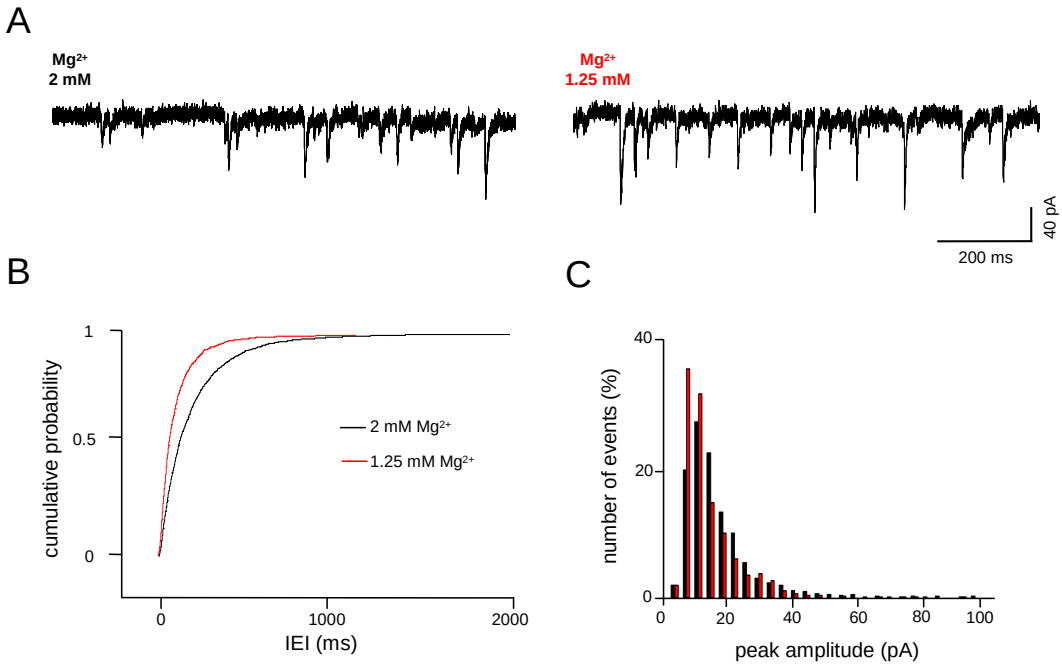


Figure 6.3. Effect of reduced $[Mg^{2+}]_o$ on sEPSCs in layer III. *A: Concurrent recordings from a typical layer III neurone in 'normal' (2 mM) and low (1.25 mM) Mg^{2+}. B: Cumulative probability graph of IEI of sEPSCs in layer III in 'normal' and low Mg^{2+}. C: Frequency distribution of sEPSC amplitudes in layer III neurones (bin width = 3). The distribution shows a single peak, skewed toward larger amplitude events.*

6.3.1.2. Effect on eEPSCs.

I next looked at the effects of lowering Mg^{2+} on AMPAr-mediated EPSCs in 7 neurones. eEPSCs were evoked using a stimulation train of 5 pulses at 10 Hz. As in the studies in Sections 4.3.1.3, 4, 5 and 6, there was a facilitation of eEPSCs between the first and the second responses, which was maintained or decreased slightly across the subsequent events in the train. Reducing $[Mg^{2+}]_o$ caused an increase in amplitudes of all events in the train (Figure 6.4C). The mean amplitude of the first eEPSC in low Mg^{2+} was 176.2 ± 15.2 pA compared to 99.3 ± 9.9 pA in control (ANOVA – $P<0.01$; n=6). During the subsequent train, amplitudes decreased slightly, but not significantly, compared to the first response. All eEPSCs remained larger than the corresponding events in control. Thus, the increase in amplitude was found to be significant at every point in the train (*P* values ranging from < 0.05 to < 0.01). Analysis of the PPR in the two conditions showed that reducing Mg^{2+} eliminates frequency-dependent facilitation between the first two responses (PPR from 1.35 ± 0.07 to 0.94 ± 0.07; Figure 6.4D) and across the rest of the train.

I carefully compared the decay kinetics of the eEPSCs to determine if slow KAr or NMDAr components were evident in low Mg^{2+}. Decay times of the first events in the trains were 51.5 ± 7.2 ms in control vs. 47.3 ±12.4 ms in low Mg^{2+}, so there was no indication that the eEPSCs were prolonged. Likewise no differences were found when comparing events at later points in the train (see Table 6.2). If anything decay times were decrease rather than prolonged. 'Before and after' analysis was also conducted in these neurones. There was no difference in the amplitude, decay times or 10-90% rise times for events recorded before or after the 10 Hz trains in either control or in low Mg^{2+}. However, under control conditions the number of events increased from 5 ± 1 before the train to 7 ± 1 after each train ($P<0.05$, n=7). In 1.25 mM Mg^{2+} the number of events before the train increased to 8 ± 1 ($P<0.05$, n=7), with no concurrent change in the number of events after the train (7± 1).

Overall, the effect of reducing $[Mg^{2+}]_o$ on excitatory transmission was very similar to the effect of applying ATPA (see Section 4.3.1.4.). sEPSC frequency but not amplitude was enhanced, and evoked responses were increased in amplitude with an overall loss of the frequency facilitation profile. Likewise, low Mg^{2+} eliminated sEPSC differences in the 'before and after' analysis, in a similar fashion to ATPA.

		1	2	3	4	5
Control	Amplitude	99.3 ± 9.9	132.0 ± 10.5	127.0 ± 14.9	123.8 ± 8.9	125.0 ± 13.8
	Decay time	51.5 ± 7.3	60.9 ± 12.6	52.4 ± 8.3	59.2 ± 11.6	57.5 ± 10.7
Low Mg^{2+}	Amplitude	176.2 ± 15.2†	165.8 ± 17.6*	164.8 ± 16.6*	160.5 ± 16.6*	152.3 ± 10.4*
	Decay time	47.3 ± 12.4	43.8 ± 11.6	45.3 ± 10.0	44.5 ± 11.3	39.6 ± 9.8

Table 6.2. Amplitude (pA) and decay times (ms) of eEPSCs in 'normal' and low Mg^{2+}.* indicates a significant difference when compared to control (*P<0.05 One way anova and Bonferroni test)*** † ***indicates a significant difference when compared to control (****P<0.01 One way anova and Bonferroni test).***

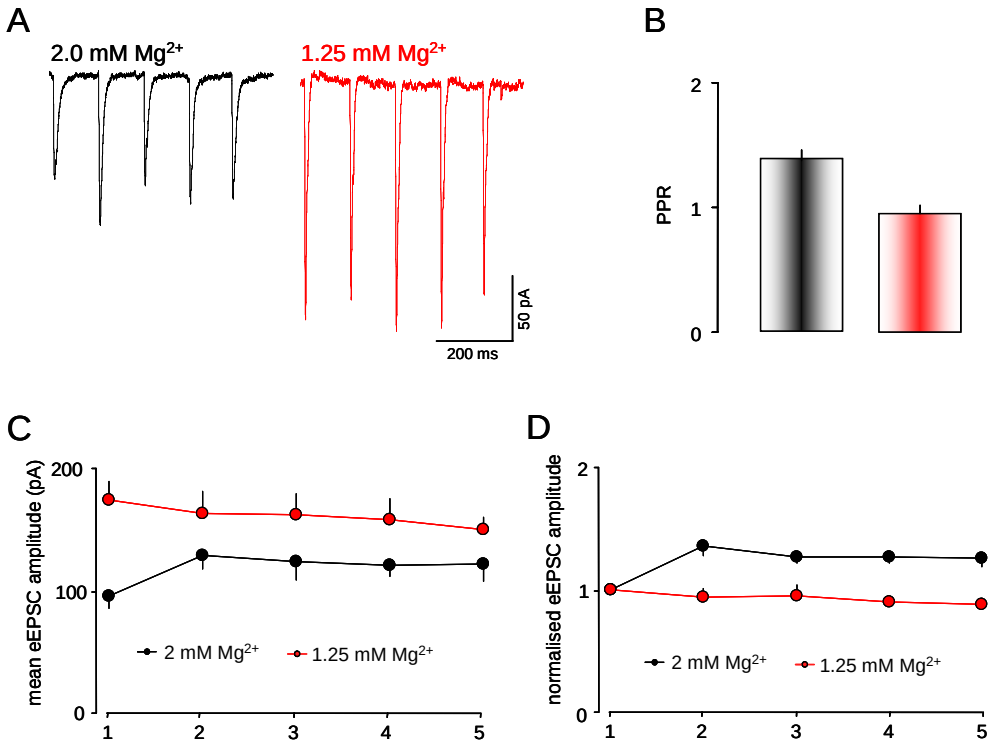


Figure 6.4. eEPSCs in 'normal' and reduced $[Mg^{2+}]_o$. *A: Consecutive recordings of eEPSCs during a 5 shock 10 Hz train in 'normal' (2 mM) and low (1.25 mM) Mg^{2+} B: PPR graph for 'normal' and low Mg^{2+} (n=6). C: eEPSC mean amplitude graph in 'normal' and low Mg^{2+}. D: Graph of normalised amplitude in 'normal' and low Mg^{2+}.*

6.3.1.3. Effects of reducing $[Mg^{2+}]_o$ is not due to NMDAr

In the next set of experiments I determined whether reducing $[Mg^{2+}]_o$ removed Mg^{2+} block and increased activation of NMDAr could lead to the increase in sEPSC frequency and eEPSC amplitude. To do this I determined the effects of the NMDAr antagonist 2-AP5 on these effects. Reducing $[Mg^{2+}]_o$ to 1.25 mM resulted in an increase in sEPSC frequency from 6.2 ± 1.0 Hz to 12.7 ± 2.1 Hz, reflecting a change

in IEI from 180 ± 31 ms to 89 ± 16 ms. After the addition of 2-AP5 (50 µM) there was a small but significant increase in IEI to 119 ± 24 ms (P<0.01; n=5). However, the change was not dramatic, and even in the presence of 2-AP5 the frequency of sEPSCs (10.9 ± 2.8 Hz) was still much higher than in 2 mM Mg^{2+} (Figure 6.5). There was, however, a significant decrease in the decay times of the sEPSCs from 8.3 ±0.6 ms to 5.0 ± 0.2 ms. This may indicate that there is some contribution of postsynaptic NMDAr to the synaptic current which is blocked by 2-AP5. IEI, amplitude and kinetics of sEPSCs are presented in Table 6.3.

sEPSC	IEI (ms)	Amplitude (pA)	Decay time (ms)	Rise Time (ms)
1.25 mM Mg^{2+}	89 ± 15	12.8 ± 1.2	8.3 ± 0.6	1.4 ± 0.2
+ 2-AP5	119 ± 24*	11.5 ± 0.7	5.02 ± 0.2	1.2 ± 0.1

Table 6.3. IEI, amplitude and kinetics for sEPSCs in reduced $[Mg^{2+}]_o$ and in the presence of 2-AP5.* indicates a significant difference when compared to control (*P*<*0.01 KS Test).***

In addition, 2-AP5 had no significant effect on the amplitude of the evoked responses (average amplitude across the train from 163.44 ± 7.69 pA to 153.39 ± 5.82 pA). However, there was a tendency for the amplitude of the responses to be smaller in the presence of 2-AP5 (Table 6.4 and Figure 6.5). There was also a tendency for the decay time of the eEPSC to decrease in the presence of 2-AP5, however this was not significant (Table 6.4).

		1	2	3	4	5
Low Mg^{2+}	Amplitude	168.7±17.8	169.0±11.7	169.5±17.7	153.6±13.6	156.4±33.1
	Decay time	20.8±4.3	20.4±0.4	21.0±0.9	24.2±6.2	20.7±2.5
+ 2-AP5	Amplitude	154.1±15.9	147.8±16.5	169.5±7.7	149.2±14.1	146.4±12.6
	Decay time	18.9±1.2	18.1±0.2	19.3±0.6	19.6±0.4	18.9±0.6

Table 6.4. Amplitude (pA) and decay times (ms) for eEPSCs in reduced $[Mg^{2+}]_o$ and in the presence of 2-AP5.

These results indicate that there may be a small contribution of NMDAr to the change in glutamate transmission induced by lowering $[Mg^{2+}]_o$. It should be remembered that 2-AP5 does not alter sEPSC frequency in normal $[Mg^{2+}]_o$ (Section 3.3.1.5. and

4.3.1.6.). The decrease seen in 1.25 mM may indicate that there is a weak activation of presynaptic NMDAr in this condition. However, this is not what is responsible for the large increase in spontaneous activity. In 2 mM Mg^{2+}, frequency-dependent facilitation of eEPSCs was blocked by 2-AP5. There was a tendency for eEPSCs to be reduced in 1.25 mM Mg^{2+}, but again this effect was small. Overall then, increased activation of NMDAr in 1.25 mM Mg^{2+} may contribute to the enhanced glutamatergic transmission but it is not responsible for the majority of the effect.

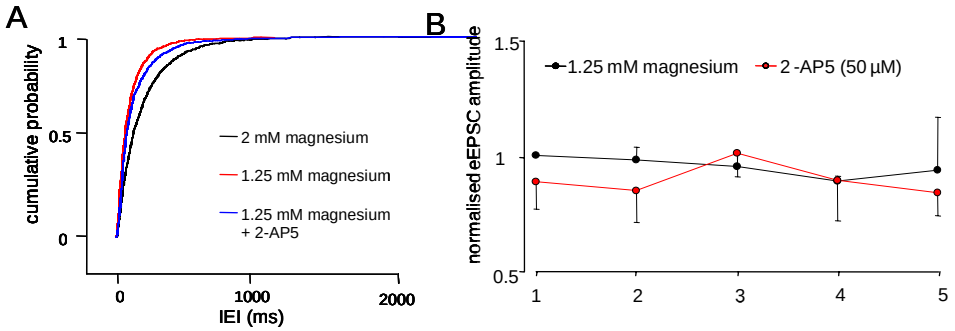


Figure 6.5. Effects of 2-AP5 on sEPSCs and eEPSCs in low Mg^{2+}. *A: Cumulative probability graph of IEI for sEPSCs in 'normal' and low Mg^{2+} conditions and after addition of 2-AP5 (50 µM) B: Normalised amplitude graph for eEPSCs in low Mg^{2+} and after addition of 2-AP5 (50 µM).*

6.3.1.4. Reducing $[Mg^{2+}]_o$ causes increased activation of GluR5 KAr

The SWO produced by reducing Mg^{2+} in the EC (Cunningham *et al.*, 2006b) can be blocked by UBP 302 so in the next set of experiments I examined the effects of the antagonist (20 µM) on the effects of lowering Mg^{2+}. As before, reducing Mg^{2+} to 1.25 mM resulted in a decreased IEI (from 192 ± 31 ms to 103 ± 19 ms; $P<0.001$; n=6) and reflecting an increase in sEPSC frequency (from 6.6 ± 0.9 Hz to 11.5 ± 2.0 Hz) similar to that described earlier. Addition of UBP 302 substantially reversed this effect, increasing inter-event interval from 103 ± 19 ms to 201 ± 37 ms (5.8 ± 1.0 Hz; KS test – $P<0.01$; n=6; Figure 6.6.), back to similar levels as in control. The data in Table 6.5 show that the event amplitudes, rise and decay times were unaltered throughout. The ability of UBP 302 to decrease sEPSC frequency in low Mg^{2+} contrasts with its failure to do so in 2 mM Mg^{2+} (Section 4.3.1.2.) i.e. this means that with the increased glutamate release seen in 1.25 mM Mg^{2+} they are now tonically activated. Thus, in this situation, the GluR5 KAr are either more sensitive to glutamate or more readily accessed by released glutamate. These possibilities are addressed below.

sEPSC	IEI (ms)	Amplitude (pA)	Decay time (ms)	Rise Time (ms)
1.25 mM Mg^{2+}	102 ± 19	14.6 ± 0.4	7.6 ± 0.6	1.4 ± 0.1
+ UBP 302	201 ± 37*	14.5 ± 1.0	7.8 ± 0.4	1.5 ± 0.1

Table 6.5. IEI, amplitude and kinetics for sEPSCs in reduced $[Mg^{2+}]_o$ and in the presence of UBP 302. * indicates a significant difference when compared to control (*P*<*0.01 KS Test).*

I have also determined the effect of UBP 302 on eEPSCs in low Mg^{2+}. As before lowering Mg^{2+} increased the amplitude of all events in a 10 Hz 5 pulse train, and essentially eliminated frequency-dependent facilitation. Figure 6.6 clearly shows that UBP 302 decreases the amplitude of all the events in the train which was significant for all positions in the train (*P* values ranging from 0.05 to 0.0001; n=3). Data for amplitudes and decay times are included in Table 6.6. It is also clear from Figure 6.6 that the reversal of the effects of Mg^{2+} was also accompanied by a lack of the frequency facilitation also seen in 2 mM Mg^{2+} after the addition of UBP 302 (Section 4.3.1.5.).

		1	2	3	4	5
Low Mg^{2+}	Amplitude	154.1±15.9	147.8±16.5	169.5±7.7	149.2±14.1	146.4±12.6
	Decay time	18.9±1.2	18.1±0.2	19.3±0.6	19.6±0.4	18.9±0.6
+ UBP 302	Amplitude	87.9±14.3†	89.1±7.5*	59.6±7.7^	94.1±5.3†	92.0±10.†
	Decay time	17.1±1.5	19.1±0.5	18.7±0.8	18.9±1.2	17.2±1.9

Table 6.6. Amplitude (pA) and decay times (ms) for eEPSCs in Low Mg^{2+} and UBP 302. * indicates a significant difference when compared to control (*P*<*0.05 ANOVA and Bonferroni test).* † ***indicates a significant difference when compared to control (****P*<*0.001 ANOVA and Bonferroni test).*^ ***indicates a significant difference when compared to control (****P*<*0.0001 ANOVA and Bonferroni test).*

I also conducted 'before and after' analysis in these neurones. There was no difference in the amplitude, decay times or 10-90% rise times for events recorded before or after the trains in either in low Mg^{2+} or in UBP 302. In low Mg^{2+} the number of events was 15.2 ± 4.4 before the train compared to 15.9 ± 4.3 after. After addition of UBP 302 the number of events before the train decreased to 7 ± 2, similarly the number of events after the train decreased to 9 ± 2. Both were significant compared to the number of events in control (*P*<0.05, n=4). Although, the number of events after the train in UBP 302 was slightly higher compared to before this was not significant.

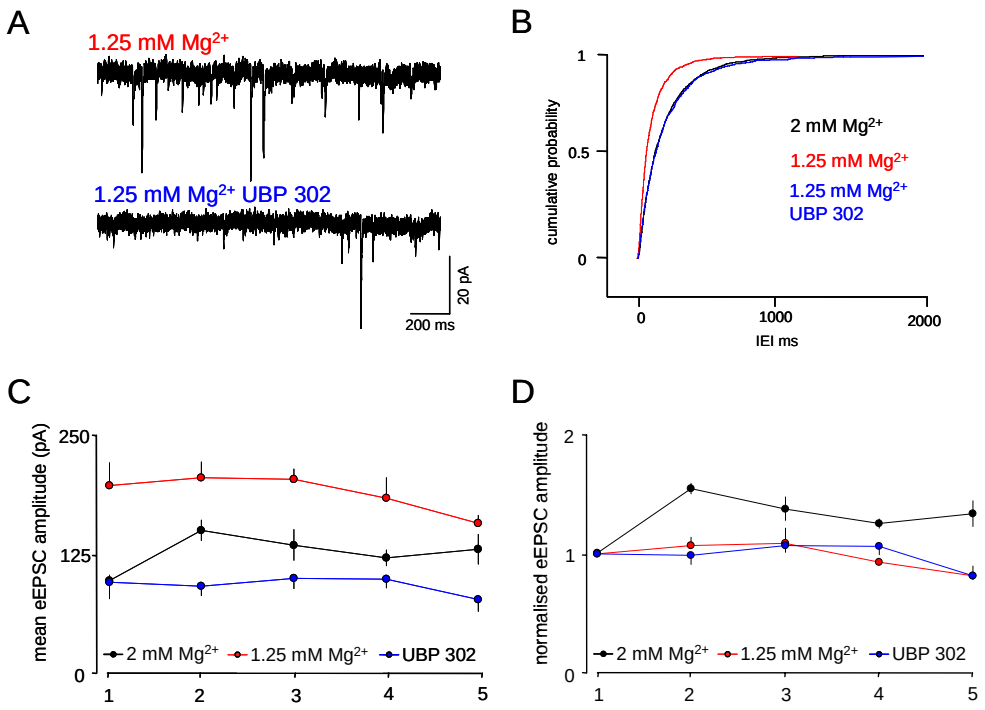


Figure 6.6. Effect of UBP 302 on sEPSCs and eEPSCs in low Mg^{2+}. *A: Concurrent recordings of sEPSCs in reduced Mg^{2+} after the addition of UBP 302 (20 µM) in a typical layer III neurone. B: Cumulative probability graph of sEPSC IEI in 'normal' and low Mg^{2+} and after addition of UBP 302 (n=6). C: Mean peak amplitude graph of eEPSCs in 'normal' and low Mg^{2+} and after addition of UBP 302 (n=4). D: Normalised amplitude graph of eEPSCs in 'normal' and low Mg^{2+} and after addition of UBP 302 (n=4).*

In summary, the data in the previous sections show that the depletion of $[Mg^{2+}]_o$ elevates transmission at glutamatergic synapses. However, these effects are only weakly ameliorated by blocking NMDAr but substantially reversed by blocking GluR5 KAr.

6.3.2 Blocking glutamate uptake mimics the effects of reducing $[Mg^{2+}]_o$

The reversal of enhanced glutamate transmission by UBP 302 suggests two main possibilities; 1, that glutamate release is increased by lowering $[Mg^{2+}]_o$ and the increased transmitter present can access KAr that were not tonically activated before. Or 2, that KAr function is somehow directly increased by removing Mg^{2+} i.e. that the receptor is somehow gated by Mg^{2+}. I have attempted to look at these possibilities. To investigate the first of these possibilities, I looked at the effects of the glutamate uptake blocker PDC. The rationale behind this is that by reducing uptake, PDC should

make more glutamate available at the synapses, which may then mimic the increased release seen with low Mg^{2+}.

6.3.2.1. Effect of PDC on sEPSCs

In 6 neurones the IEI of sEPSCs was 178 ± 33 ms. Application of PDC (50 µM) significantly decreased this to 92 ± 17 ms (P<0.0001; n=6). These results reflect an average change in frequency from 6.8 ± 3.3 Hz to 14.3 ± 3.2 Hz. Kinetics of the events were unaltered (Table 6.7). However, PDC also caused a small increase in the amplitude of the sEPSCs from 14.4 ±1.9 pA to 17.1 ± 2.0 pA (P<0.05; n=6). Thus, blocking uptake resulted in an increase in frequency, which likely reflects an increased probability of release. At the same time the increased amplitude may indicate that the postsynaptic receptors may not be saturated, and each release event can now reach more receptors. Thus, the increase in glutamate release is similar to that elicited by lowering $[Mg^{2+}]_o$. I then tested the effects of UBP 302 on sEPSC. Addition of the antagonist largely blocked the change in frequency induced by PDC reducing the IEI back to 147 ± 21 ms (P<0.001; n=6; Figure 6.7.). However, it did not completely reverse it, and the frequency (7.6 ± 1.2 Hz) remained elevated over control levels. Average amplitude was decreased to 15.4 ± 1.4 pA, although this was not found to be significant.

sEPSC	IEI (ms)	Amplitude (pA)	Decay time (ms)	Rise Time (ms)
Control	178 ± 33	14.4 ±1.9	9.3 ± 1.0	1.9 ± 0.2
+ PDC	92 ± 17*	17.1 ± 2.0†	9.7 ± 1.7	1.7 ± 0.3
+ UBP 302	147 ± 21^	15.4 ± 1.4	8.5 ± 0.6	1.6 ± 0.2

Table 6.7. IEI, amplitude and kinetics for sEPSCs in PDC and UBP 302. **indicates a significant difference when compared to control (*P*<0.0001 KS Test). †indicates **a significant difference when compared to control (P<**0.05 ANOVA and Bonferroni test). ^ inidicates a significant difference when compared to UBP 302 ((*P*<0.001 KS Test**).***

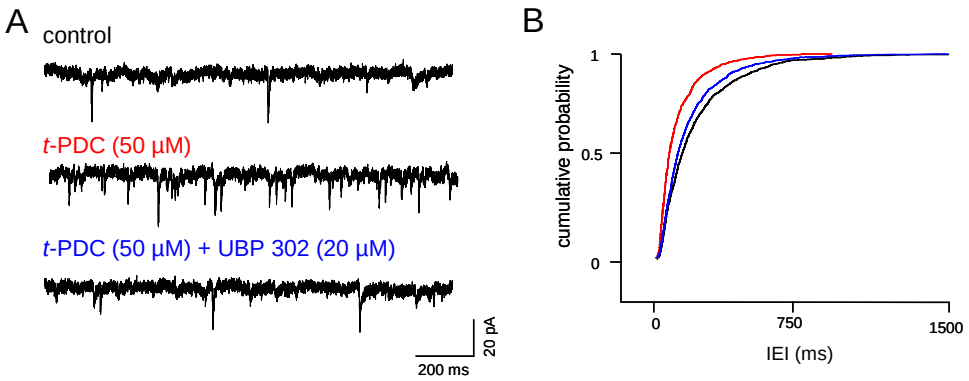


Figure 6.7. Effects of PDC on sEPSCs. *A: Consecutive recordings of sEPSCs in control conditions and after addition of PDC (50 µM) and UBP 302 (20 µM). B: Cumulative probability graph of IEI in control conditions and after the addition of PDC and UBP 302 (n=6).*

6.3.2.2. Effects of PDC on eEPSCs

I also determined the effect of PDC on eEPSCs. As with lowering Mg^{2+}, PDC (50 µM) increased the amplitude of all events in a 5 Hz 10 pulse train, and essentially eliminated frequency-dependent facilitation. Figure 6.8 clearly shows that PDC increases the amplitude of all the events in the train and this was significant for all positions in the train (*P* values ranging from 0.05 to 0.01; n=4). Data for amplitudes and decay times are included in Table 6.8. It is also clear from Figure 6.8 that PDC also removes the PPF seen in control. I then tested the effects of UBP 302 (20 µM) on eEPSCs in these neurones. UBP 302 significantly reduced the amplitude of all events in the train (*P* values ranging from 0.05 to 0.01) without affecting decay times (Table 6.8 and Figure 6.8). There is also no PPF in UBP 302, as was seen in Section 4.3.1.5.

In 'before and after' analysis there was no difference in the amplitude, decay times or 10-90% rise times for events recorded before or after the trains in either in PDC (50 µM) compared to control. In control the number of events increased from 8.4 ± 1.6 before the train to 11.4 ± 2.0 after each train ($P<0.05$, n=6). However, after addition of PDC the number of events before the train was increased overall to 10.7 ± 2.0 ($P<0.05$, n=6), but there was no concurrent change in the number of events after the train (11.9 ± 1.9) when the blocker was present.

		1	2	3	4	5
control	Amplitude	79.5 ± 13.9	116.5 ± 8.6	119.7 ± 11.4	137.7 ± 7.3	151.3 ± 13.0
	Decay time	10.2 ± 1.2	10.0 ± 1.3	9.9 ± 1.0	10.3 ± 0.9	10.3 ± 0.7
+ PDC	Amplitude	201.5 ± 30.2*	190.3 ± 25.1†	215.3 ± 32.8†	228.1 ± 34.6†	246.3 ± 44.8†
	Decay time	9.4 ± 0.4	9.3 ± 0.5	9.2 ± 0.5	9.2 ±0.5	9.1 ± 0.5
+ UBP 302	Amplitude	66 ± 14.5^	63.6 ± 13.8†^	59.2 ± 13.6^{+}	74.3 ± 14.6^	69.4 ± 15.3^
	Decay time	9.8 ± 0.8	10.5 ± 0.7	10.8 ± 1.9	10.3 ± 0.7	10.8 ± 1.0

Table 6.8. Effects of PDC on eEPSC amplitude (pA) and decay time (ms).* indicates a signidicant difference when compared to control (*P*** *<0.01 ANOVA and Bonferroni test).* † ***indicates a signidicant difference when compared to control (****P*** *<0.51 ANOVA and Bonferroni test).* ^***indicates a signidicant difference when compared to PDC (****P*** *<0.05 ANOVA and Bonferroni test).* $^{+}$***indicates a signidicant difference when compared to PDC (****P*** *<0.05 ANOVA and Bonferroni test).***

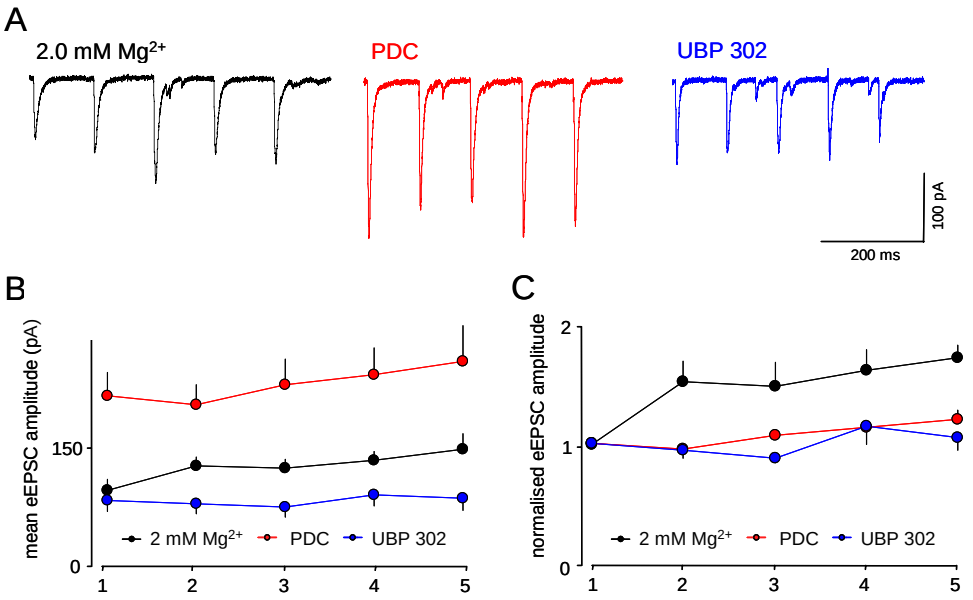


Figure 6.8. Effects of PDC on eEPSCs. *A: Concurrent recordings average eEPSCs in control conditions (2 mM* Mg^{2+}*) and in the presence of PDC (50 µM) and UBP 302 (20 µM) in a typical layer III neurone. B: eEPSC mean amplitude graph (n=4) showing control conditions and after addition of PDC and UBP 302. C: eEPSC normalised amplitude graph (n=4) showing control and after addition of PDC and UBP 302.*

Thus, in essence, the effects of reducing $[Mg^{2+}]_o$ were mimicked by blocking glutamate uptake. This included the ability of GluR5 receptor blockade to at least partially reverse the increase in spontaneous glutamate transmission. This strongly

suggests that the increased glutamate release is boosted by activation of KAr receptors, which are not normally accessed by ambient glutamate.

6.3.3. Postsynaptic KAr in low Mg^{2+} and PDC

The previous experiments suggested that presynaptic KAr function was increased in low Mg^{2+} and during application of PDC. In a further series of experiments I looked at changes in postsynaptic KAr eEPCSs induced by reducing $[Mg^{2+}]_o$ or blocking uptake. In these experiments I used a stimulation protocol of 5 pulses at 100 Hz in the presence of GYKI 53665 and 2-AP5, which elicited a small amplitude, slowly decaying EPSC mediated by KAr (Section 4.3.2.).

I found that reducing $[Mg^{2+}]_o$ from 2 mM to 1.25 mM significantly increased the amplitude of the KAr eEPSCs from 60.5 ± 6.9 pA to 84.9 ± 6.5 pA ($P<0.01$; n=6; Figure 6.9A). The kinetics of the event were unaltered, however, with a decay time of 675.4 ± 58.2 ms in control compared to 711.6 ± 50.6 ms in low Mg^{2+}.

There are a number of possible explanations for this increase in amplitude, including increased release of glutamate, or a change in the postsynaptic receptor. In Section 4.3.2.1. I showed that the KAr eEPSC was likely to be mediated by a KAr subunit other that GluR5 as it wasn't blocked by UBP 302. The I-V relationship of the EPSC suggested that it might be an edited version of a GluR6 containing receptor with low Ca^{2+} permeability (Section 4.3.2.3.). It has been shown that GluR6 receptors can be weakly blocked by Mg^{2+} (Fukushima *et al.*, 2003). One possibility is that the reduction of $[Mg^{2+}]_o$ increased current through the postsynaptic receptors. This might be expected to result in a change in I-V relationship, so I have examined whether there was any effect of reducing Mg^{2+} on the I-V curve of the KAr eEPSC. It is clear from Figure 6.9B that reducing $[Mg^{2+}]_o$ had no effect on the I-V relationship of the receptor which supports the suggestion that there is no direct effect of Mg^{2+} on the postsynaptic KAr itself.

Since the effects of low Mg^{2+} on sEPSCs and eEPSCs was reversed by UBP 302, another possibility is that the KAr EPSC is enhanced by additional contribution from GluR5 receptors. To test this I looked at the effects of UBP 302 on this increase in the eEPSC amplitude in all six neurones where the KAr eEPSC was enhanced in low

Mg^{2+}. UBP 302 caused a decrease in the amplitude of the KAr eEPSC from 84.9 ± 6.5 pA to 64.3 ± 3.9 pA (P<0.01; n=6), i.e. virtually the same as the amplitude in control (60.5 ± 6.9 pA). There was no change in kinetics of the eEPSC in the presence of UBP 302 (687.2 ± 96.5 ms). Thus, reducing $[Mg^{2+}]_o$ appeared to induce a GluR5 mediated component to the KAr EPSC that was not present under control conditions (see Section 4.3.2.1.). The results from studies on sEPSCs and AMPAr-mediated eEPSCs above suggest that the most likely explanation is that low Mg^{2+} increases release of glutamate to then tonically activate presynaptic autoreceptors.

Results obtained from blocking glutmate uptake agree with this scenario. I examined the effects of PDC on the KAr eEPSC in 2 mM Mg^{2+}. Application of PDC (50 µM) caused an increase in the amplitude of the KAr eEPSC from 65.9 ± 6.6 pA to 100.3 ± 12.6 pA (P<0.05; n=7), with no concurrent change in decay time (from 529.2 ± 49.60 ms to 541.5 ± 68.0 ms). Again this increase was largely, although not completely, reversed in the presence of UBP 302 (75.9 ± 6.8 pA; P<0.05; n=6; Figure 6.9C). Surprisingly perhaps, there was no change in kinetics of the eEPSC in the presence of UBP 302 (500.5 ± 48.4 ms).

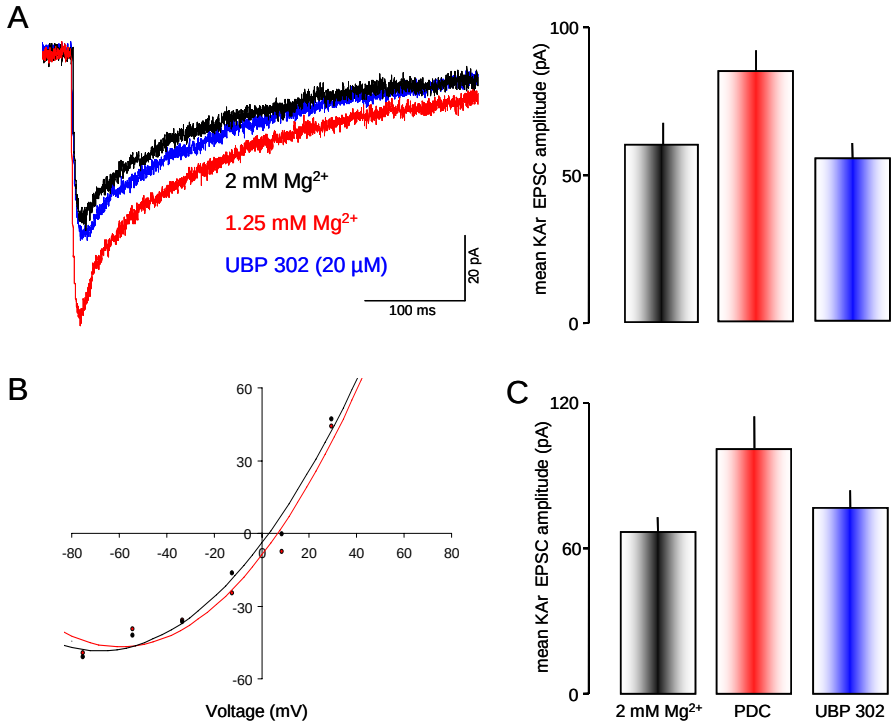


Figure 6.9. Postsynaptic KAr EPSC in reduced $[Mg^{2+}]_o$. *A: Consecutive recordings of KAr eEPSCs in 'normal' (2 mM) and low Mg^{2+} (1.25 mM) and after addition of UBP 302 (20 µM) and mean amplitude graph (n=6) in 'normal' Mg^{2+} and low Mg^{2+} and after addition of UBP 302. B: Current-Voltage plot (n=4; corrected for LJP). C: Mean amplitude graph (n=6) in 'normal' Mg^{2+} and after addition of PDC (50 µM) and UBP 302.*

6.3.4 Effects of reducing $[Mg^{2+}]_o$ on GABAergic transmission

The foregoing experiments clearly indicate an enhanced level of excitatory transmission under conditions that induced SWO. This enhancement is at least partly mediated by GluR5 receptors. These receptors also control aspects of inhibition (Chapter 5) and increased GABAergic inhibition is important in SWO (Sanchez-Vives and McCormick, 2000). Therefore, I also examined GABA transmission during the lowering of $[Mg^{2+}]_o$ that can elicit SWO (Cunningham *et al.*, 2006b).

6.3.4.1. Effect on sIPSCs

In 6 neurones (in the presence of GYKI 53665 and 2-AP5 to block AMPAr and NMDAr respectively) the mean IEI of sIPSCs was 167 ± 32 ms. As was the case with sEPSCs, reducing the $[Mg^{2+}]_o$ from 2 mM to 1.25 mM caused a large decrease in IEI to 88 ± 11 ms (KS test – $P<0.0001$; n=6, see Figure 6.10) reflecting an increase in frequency from 7.5 ± 4.2 Hz to 12.1 ± 3.2 Hz. Concurrently, the amplitude of the sIPSCs was also increased. There was a tendency for the decay times to be longer, but this change was not significant. Rise times were unaltered.

In view of the fact that UBP 302 reversed the effects of lowering Mg^{2+} on EPSCs, I next examined the effect of the antagonist on the increase in sIPSC frequency induced by low Mg^{2+} in a further 4 neurones. In these neurones IEI was 138 ± 31 ms in 2 mM Mg^{2+} and this showed a substantial decrease to 52 ± 5 ms when Mg^{2+} was reduced to 1.25 mM. Addition of UBP 302 (20 μM) then increased IEI from this reduced level to 78 ± 11 ms (KS test – $P<0.0001$; n=4). This was a partial reversal, as IEI did not return to control levels (KS test $P<0.001$; n=4; see Figure 6.10). This indicated that, as with sEPSCs, increased GluR5 receptor activation contributes to the increase in inhibition.

However, as UBP 302 is specific for GluR5 receptors, I also wanted to determine whether other KAr may be involved. Thus, subsequent addition of CNQX (20 μM) was made. This resulted in a further increase in IEI to 86 ± 12 ms ($P<0.05$; n=4; see Figure 6.10) but again this was still significantly shorter than in control ($P<0.001$; n=4). Average amplitudes and kinetics of sIPSCs in 'normal' and low Mg^{2+} and after

addition of UBP 302 and CNQX is summarised in Table 6.9. It can be seen that the increase in amplitude elicited in 1.25 Mg^{2+} is also reversed in parallel with IEI.

sIPSC	IEI (ms)	Amplitude (pA)	Decay time (ms)	Rise Time (ms)
2.0 mM Mg^{2+}	138 ± 31	34.4 ± 2.4	8.6 ± 0.5	1.9 ± 0.4
1.25 mM Mg^{2+}	52 ± 5*	38.5 ± 3.0	10.4 ± 1.7	1.6 ± 0.2
+ UBP 302	78 ± 11$^{\dagger\wedge}$	37.1 ± 2.2	9.2 ± 0.7	1.8 ± 0.3
+ CNQX	86 ± 12$^{\wedge+}$	35.4 ± 4.4	10.0 ± 0.8	1.9 ± 0.3

Table 6.9. IEI, Amplitudes (pA), decay and rise times (ms) for sIPSCs in high and low Mg^{2+} and in the presence of UBP 302 and CNQX. * indicates a signidicant difference when compared to control (*P<0.0001 ANOVA and Bonferroni test). † ***indicates a signidicant difference when compared to low magnesium (****P<0.0001 ANOVA and Bonferroni test). ^**indicates a signidicant difference when compared to control (****P<0.001 ANOVA and Bonferroni test). $^{+}$**indicates a signidicant difference when compared to UBP 302 (****P<0.05 ANOVA and Bonferroni test).***

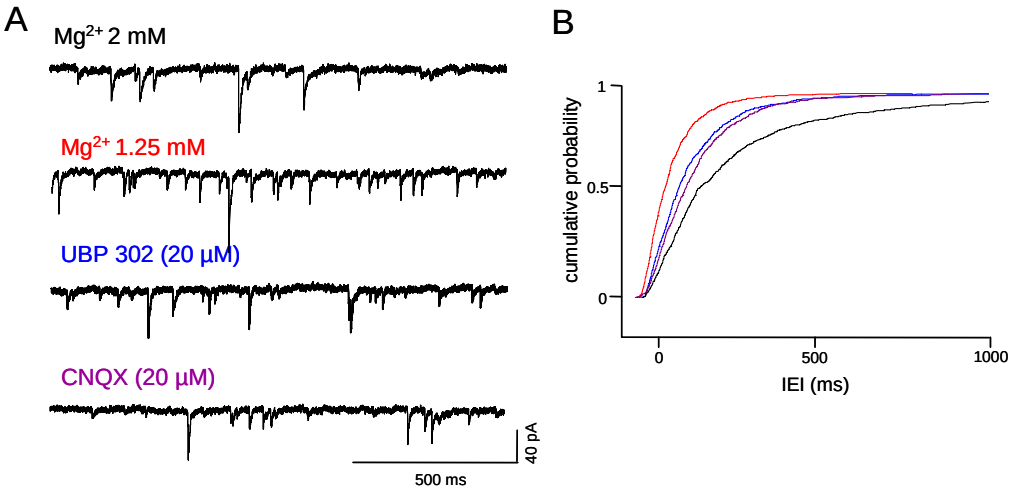


Figure 6.10. Effect of reducing $[Mg^{2+}]_o$ on sIPSCs. *A: Concurrent recordings of sIPSCs in 'normal' and low Mg^{2+} and after addition of UBP 302 and CNQX. B: Cumulative probability graph of IEI of sIPSCs in 'normal' (2 mM) and low Mg^{2+} (1.25 mM) and after perfusion with UBP 302 (20 µM) and CNQX (20 µM).*

6.4.3.2. Effects on eIPSCs

I have also examined changes in $GABA_A$r-mediated eIPSCs during lowering of $[Mg^{2+}]_o$. Again, I employed 5 pulse trains at 10 Hz to evoke the inhibitory responses. As before, there was a clear PPD between the first and second responses followed by a plateau (Section 5.3.3.1.). Reducing $[Mg^{2+}]_o$ resulted in an increase in amplitudes of

all eIPSCs in the train. The average overall amplitude of eIPSCs across the train in low Mg^{2+} was 366.0 ± 32.7 pA compared to 209.4 ± 21.5 pA in control (P<0.0001, n=6). The increase in amplitude was found to be significant wherever the event was in the train (P values ranging from 0.05 to 0.01; Figure 6.11). Despite the elevated amplitudes the overall profile of eIPSCs in the train was little affected and this was shown by a lack of change in PPD (Figure 6.11). There was a tendency for the decay time of eIPSCs to increase but it was not significant (from 38.7 ± 14.0 ms to 53.3 ± 12.3 ms).

Thus, as with effects on eEPSCs, lowering $[Mg^{2+}]_o$ results in a large increase in amplitude of all evoked responses, which bears a strong similarity to the effects of perfusing with ATPA (see Section 5.3.3.2.). For this reason I have also studied the effect of KAr blockade on low Mg^{2+}-induced changes in eIPSCs. Again, low Mg^{2+} induced an increase in amplitude of all responses in a 5 Hz train, with no real change in the overall pattern of frequency-dependent depression. The average amplitude of all responses in the train increased from 217.6 ± 25.5 pA in control to 351.26 ± 21.2 pA in low Mg^{2+}. UBP 302 then decreased the average amplitude of all evoked IPSCs in the train to 238.0 ± 16.0 pA (P<0.001; n=5), and this reduction was significant for all positions in the train except the second (P values ranging from <0.05 to <0.01; Figure 6.11). Subsequent addition of CNQX (20 μM) further reduced the overall average amplitude of eIPSCs (to 153.9 ± 9.6 pA). However, only the second response in the train decreased significantly (from 260.6 ± 32.5 pA to 173.5 ± 13.2 pA; P<0.05; n-4).

The effects of the two antagonists on the profile of eIPSCs in the train were quite complex, but remarkably similar to their effects in 2 mM (Section 5.3.3.2. and 3.). Thus, UBP 302 decreased the first response in the train to below control levels, and the second response became larger, converting PPD to PPF. This meant that UBP 302 caused a significant shift on the PPR (from 0.7 ± 0.1 to 1.4 ± 0.1; P<0.05; n=5; Figure 6.11D). When CNQX was also added the initial PPF now disappeared, and the response profile became flat, with a consequent PPR of 1.1 ± 0.1 (P<0.05; n=5; see figure 6.11D). The average amplitude of evoked responses after CNQX application was also significantly less than in 2 mM Mg^{2+} (217.2 ± 25.5; P<0.05; n=4), although the only IPSC in the train that was significantly decreased was the second response (from 281.2 ± 84.9 to 169.1 ± 29.4; P< 0.05; n=4; Figure 6.11).

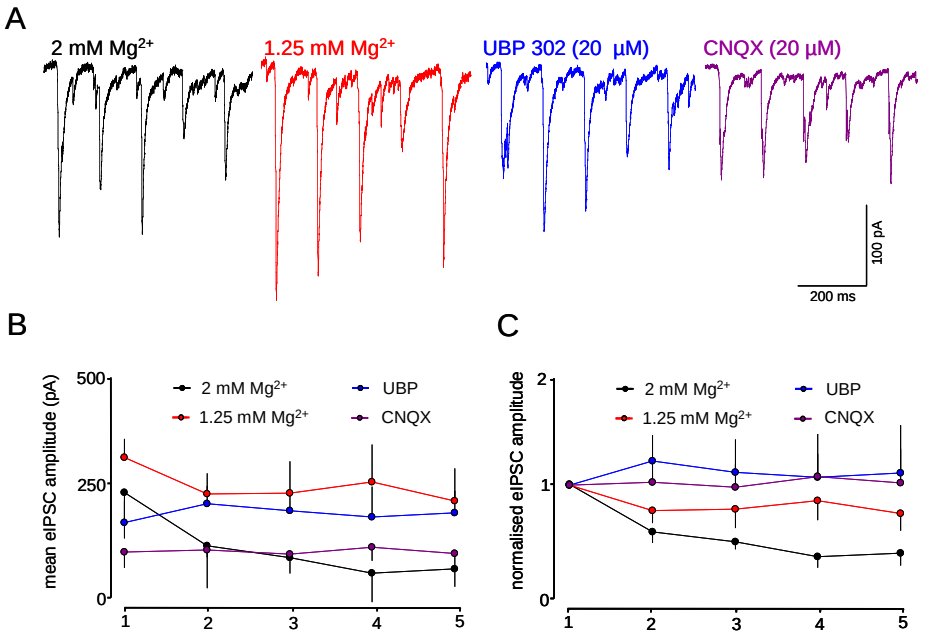


Figure 6.11. Effects of reducing $[Mg^{2+}]_o$ on eIPSCs. *A: Consecutive recordings of eIPSCs at 10 Hz in 'normal' (2 mM) and low Mg^{2+} (1.25 mM) and after addition of UBP 302 (20 µM) and CNQX (20 µM). B: eEPSC mean amplitude graphs (n=5) in 'normal' and low Mg^{2+} and after addition of UBP 302 and CNQX. C: Normalised amplitude graphs (n=5) of eIPSCs in 'normal' and low Mg^{2+} and after addition of UBP 302 and CNQX.*

6.3.3.3. Effect of PDC on IPSCS

Previously, I have shown that blocking glutamate uptake, using PDC, mimicked the effects of reducing $[Mg^{2+}]$ on glutamatergic transmission. This supported the conclusion that lowering Mg^{2+} caused increased glutamate release, which increased activation of GluR5 KAr at glutamatergic synapses. For completeness, I also investigated the effects of PDC on sIPSCs and eIPSCs. In 5 neurones the IEI of sIPSCs was 117 ± 23 ms. Application of PDC (50 µM) significantly decreased this to 76 ± 18 ms (P<0.0001; n=6; Figure 6.12A,B). These results reflect an average change in frequency from 10.3 ± 2.3 Hz to 16.9 ± 4.3 Hz. Amplitude and kinetics of the sIPSCs were unaltered (Table 6.10). Thus, blocking glutamate uptake resulted in an increase in sIPSC frequency similar to that caused by lowering $[Mg^{2+}]_o$. I then tested the effects of UBP 302 on sIPSC frequency in these neurones. Addition of the antagonist partially reversed the increase in frequency induced by PDC reducing the IEI back to 99 ± 18 ms (P<0.001; n=6; Figure 6.12A,B). However, it did not completely reverse it, and the frequency (11.8 ± 2.3 Hz) remained elevated over control levels.

sEPSC	IEI (ms)	Amplitude (pA)	Decay time (ms)	Rise Time (ms)
Control	117 ± 23	22.5 ± 2.5	10.2 ± 2.3	2.8 ± 0.6
+ PDC	76 ± 18*	21.5 ± 2.7	9.0 ± 3.4	2.3 ± 0.5
+ UBP 302	99 ± 18^	19.0 ± 4.9	9.7 ± 3.0	2.4 ± 0.2

Table 6.10. IEI, amplitude and kinetics for sIPSCs in PDC and UBP 302.* indicates a significant difference from control (*P<0.0001 KS Test). ^ indicates a significant difference from PDC (*P<0.001 KS Test).*

I also determined the effect of PDC on eIPSCs. As with lowering Mg^{2+}, PDC (50 µM) increased the overall average amplitude of eIPSCs in a 5 Hz 10 pulse train from 108.0 ± 11.1 pA to 180.4 ± 15.1 pA (*P*<0.0001; n=5) with no concurrent effect on PPD (from 0.52 ± 0.10 to 0.63 ± 0.06). Figure 6.12C clearly shows that PDC increases the amplitude of all the events in the train and this was significant for all positions in the train (*P* values ranging from 0.05 to 0.01; n=5). I then tested UBP 302 (20 µM) on eIPSCs in these neurones. UBP 302 significantly reduced the amplitude of the first event in the train (from 228.5 ± 48.2 pA to 116.1 ± 32.6; *P*<0.01; n=5). There was a tendency for all of the subsequent events in the train to decrease in the presence of UBP 302, however this was only significant for the fourth and fifth responses (Figure 6.12C). UBP 302 had no effect on the decay times (Table 6.10). As in Section 5.3.3.3. UBP 302 also changed the profile of responses from PPD to PPF (Figure 6.12D).

		1	2	3	4	5
control	Amplitude	137.2 ± 30.6	79.3 ± 30.3	96.0 ± 15.6	116.9 ± 17.3	110.5 ± 28.7
	Decay time	14.5 ± 3.6	14.5 ± 3.5	15.1 ± 3.7	15.4 ± 3.3	15.3 ± 2.8
+ PDC	Amplitude	228.5 ± 48.2^	161.2 ± 37.9^	161.5 ± 28.3^	188.9 ± 21.9*	161.7 ± 30.6*
	Decay time	14.3 ± 0.8	14.1 ± 0.9	14.9 ± 1.0	14.1 ± 1.0	14.7 ± 0.7
+ UBP 302	Amplitude	116.1 ± 32.6^{+}	129.1 ± 30.5	123.2 ± 20.0	125.1 ± 32.5†	109.1 ± 29.2†
	Decay time	14.8 ± 1.9	14.1 ± 1.4	14.0 ± 2.3	14.4 ± 1.6	14.7 ± 3.5

Table 6.11. Effects of PDC on eIPSC amplitude (pA) and decay time (ms) * indicates a significant difference from control (*P<0.01 KS Test). ^indicates a significant difference from control (*P<0.05 KS Test). $^{+}$***indicates a significant difference from PDC (****P<0.01 KS Test). † *indicates a significant difference from PDC (*P<0.05 KS Test).*

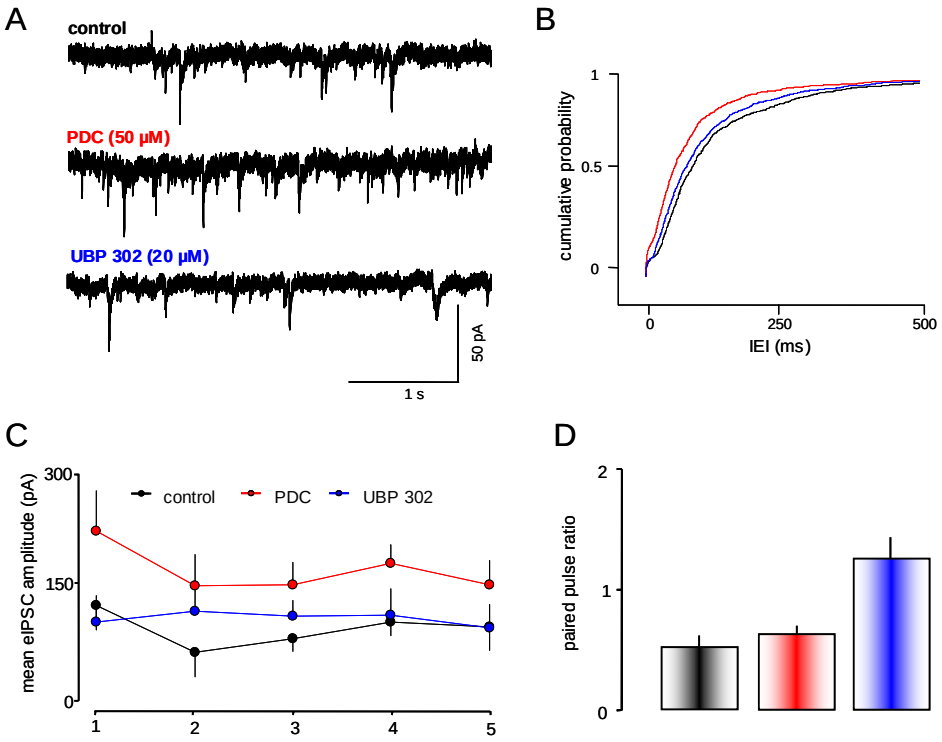


Figure 6.12. Effects of PDC on sIPSCs and eIPSCs. *A: Concurrent recordings of sIPSCs in control conditions (2 mM Mg^{2+}) and after PDC (50 µM) and UBP 302 (20 µM). B: Cumulative probability graph of sIPSC IEI in control conditions and after application of PDC and UBP 302 (n=6). C: eIPSC mean amplitude graphs showing control conditions and after perfusion of PDC and UBP 302 (n=5). D: Graph of PPR (n=5).*

Thus, in essence, the effects of reducing $[Mg^{2+}]_o$ were mimicked by blocking glutamate uptake. This included the ability of GluR5 receptor blockade to at least partially reverse the increase in spontaneous glutamate transmission. This strongly suggests that the increase in GABA transmission is at least partly due to the increase in glutamate release elicited by low Mg^{2+} which is boosting activation of the GluR5 KAr receptors which control GABA release.

6.4. Discussion

SWO can be induced in layer III of the EC when $[Mg^{2+}]_o$ is moderately reduced (Cunningham *et al.*, 2006b; Greenhill and Jones, unpublished). The aim of this chapter was to determine what this manipulation does to excitatory and inhibitory transmission in layer III and to relate this to the role of KAr. The results clearly show that when $[Mg^{2+}]_o$ is reduced there is a substantial increase in spontaneous release at glutamatergic synapses, and a concurrent increase in amplitude of the eEPSCs, both

those mediated by AMPAr and KAr. This may not be surprising as the ability of Mg^{2+} to block VGCC has long been known. A reduction in $[Mg^{2+}]_o$ might be expected to reduce a "physiological" block of VGCC in terminals and lead to increased glutamate release (Lin *et al.*, 2002; Zhang *et al.*, 1996). This could occur by two mechanisms. 1, a direct reduction of Mg^{2+} block of the VGCC poor; 2, a surface charge screening effect, where depletion of Mg^{2+} allows more Ca^{2+} to move closer to the mouth of the VGCC (Muller and Finkelstein, 1974).

In addition, presynaptic NMDAr are present in the EC and act to facilitate glutamate release (Berretta and Jones, 1996b; Woodhall *et al.*, 2001a). As NMDAr are blocked by Mg^{2+}, another possibility is that the increased glutamate release could be due to an increased activation of presynaptic NMDAr when $[Mg^{2+}]_o$ is lowered. The contribution of presynaptic NMDAr proved to be minor however, as when I applied 2-AP5, the frequency of events was reduced slightly. It was by no means abolished, therefore increased presynaptic NMDAr activation was not responsible for the large majority of the increased release observed in low Mg^{2+}. In addition, 2-AP5 had no real effect on the amplitude of the eEPSCs. This leads to the conclusion that although reducing the Mg^{2+} may cause a slight increase in presynaptic NMDAr activation other mechanisms account for the increase in spontaneous excitatory activity seen in 1.25 mM Mg^{2+}. This could be accounted for simply by the increased Ca^{2+} entry via VGCC noted above. However, as the SWO induced by lowering $[Mg^{2+}]_o$ (Cunningham *et al.*, 2006b) are blocked by UBP 302, the effect of the antagonist on the increased glutamate release was tested. It caused a substantial reversal of the increased sEPSC frequency and increase eEPSC amplitude seen in 1.25 mM Mg^{2+}. Thus, a large part of the increased release was GluR5 KAr-dependent. It should be remembered from Chapter 4 that these receptors can presynaptically facilitate release at layer III synapses, but only during repetitive stimulation. However, in 1.25 mM Mg^{2+}, the GluR5 receptors become tonically active, as UBP 302 now reduces sEPSC frequency. Thus, I suggest that glutamate release is increased by reduced blockade of VGCC and this increased release is then boosted by enhanced activation of the presynaptic GluR5 receptors.

An alternative possibility is that Mg^{2+} has a direct effect on the GluR5 KAr and that lowering Mg^{2+} increases intrinsic activity of the receptor. I checked whether this might be the case by examining the effects of PDC. Blocking glutamate uptake would

be expected to increase ambient extracellular glutamate, without having direct effects on cellular, terminal or channel excitability. PDC, like 1.25 mM Mg^{2+}, caused an increase in sEPSC frequency and an increase in the amplitude of the AMPAr-mediated EPSCs. This was expected as by blocking uptake of glutamate in the synapse it increases the availability of the transmitter to activate the postsynaptic receptors. What was interesting is that UBP 302 again substantially reversed this effect of PDC. This would indicate that the impairment of glutamate clearance from the synapse again leads to an increased activation of the presynaptic GluR5 receptors, which in turn causes even more glutamate to be released. There is a precedent for increased activation of presynaptic glutamate receptors caused by increased ambient glutamate levels after glutamate transporter blockade. In cultured hippocampal neurones, PDC actually reduced sEPSCs and this was suggested to result from increased glutamate access to presynaptic mGluR (Maki *et al.*, 1994). In fact, Iserhot *et al.* (1999) also reported that PDC at very high concentrations (400 µM) reduced excitatory field potentials in layer III and layer V in EC slices. They speculated that this could be due to enhanced activation of high affinity KAr. These results are obviously at odds with my studies. However, the authors did use very high concentrations of the blocker, which may well have direct effects on ion channels and cause desensitisation of postsynaptic AMPAr.

My studies clearly suggest that preventing glutamate uptake enhances, rather than reduces excitatory transmission and the increase in ambient glutamate boosts itself by activating presynaptic GluR5 receptors. As PDC had essentially the same effect on glutamatergic transmission as reducing Mg^{2+}, it seems unlikely that Mg^{2+} has any direct effects on the presynaptic KAr. I cannot completely rule out the possibility that PDC and low Mg^{2+} increase glutamate release, which then accesses <u>postsynaptic</u> GluR5 KAr, not normally activated by ambient or evoked glutamate release. Both manipulations caused an increase in the KAr EPSC and PDC also slightly increased sEPSC amplitude, all effects reversed by some extent by UBP 302. However, the studies in 2 mM $[Mg^{2+}]_o$ (Section 4.3.1.) strongly indicated a presynaptic GluR5 KAr. Also, low Mg^{2+} increase sEPSC frequency with <u>no</u> effect on amplitudes or kinetics. Thus, overall my conclusion is that the effects are almost certainly due to increased presynaptic GluR5 activity.

I also examined the consequences of reducing $[Mg^{2+}]_o$ on GABAergic transmission. Reduction of Mg^{2+} in the aCSF caused a pronounced increase in sIPSC frequency and an increase in amplitude of the eIPSCs, although it had no effect on PPR. Application of UBP 302 partially reduced the frequency of sIPSCs, subsequent addition of CNQX caused a further reduction although still not to the frequency seen in control. So what is the basis of the increase in GABA release and how does this relate to KAr-mediated control? It seems likely that increased GABA will occur partly due to removal of a Mg^{2+} block on the presynaptic VGCC on the GABAergic terminal. However, as both UBP 302 and CNQX have reduced frequency of sIPSCs in 'normal' Mg^{2+} (Section 5.3.1.3.), and then had a similar effect in low Mg^{2+} it is unclear from this data that there is increased activation of KAr at GABAergic synapses. However, both antagonists also reduced eIPSC amplitude, and although GABA release was greatly elevated, overall their effects were similar in both low and normal Mg^{2+}. Thus, UBP 302 decreased the amplitude of all evoked responses during repetitive stimulation, in addition to changing PPD to PPF. Importantly, the first response in the train was also reduced compared to control, whether 2 mM or 1.25 mM Mg^{2+} was used. This would indicate that in low Mg^{2+} there is an increased activation of the GluR5-containing KAr, which I suggested in the previous Chapter were postsynaptic KAr acting to drive the interneurons. Thus, low Mg^{2+} may partly increase GABA release at synapses on the interneurones where postsynaptic GluR5 receptors are located.

CNQX also decreased eIPSC amplitude, but as in 'normal' studies (see Section 5.3.3.3.) the first response in the train was not reduced compared to that seen in the presence of UBP 302. I suggested in Chapter 5 that the facilitation in eIPSCs seen in the presence of 2 mM Mg^{2+} and UBP 302 was mediated by glutamate activating presynaptic GluR6/KA2 receptors on GABA terminals. This mechanism is still active in 1.25 mM Mg^{2+}, as evidenced by the effect of CNQX, but it unclear as to whether there is increased activity of the presynaptic KAr on GABAergic terminals when $[Mg^{2+}]_o$ is reduced.

Studies with PDC in IPSCs largely reflected what was seen with low Mg^{2+}. The increase in sIPSC frequency and eIPSC amplitudes were partially reversed by UBP 302. This would fit well with these effects being partly due to increased glutamate levels present at synapses on the interneurones, driving increased GABA release via GluR5 receptors. Vignes (2001) showed in cultured hippocampal neurones that

GABA release was driven partly by AMPAr and partly by GluR5 KAr on interneurones. However, the effect of GluR5 receptors was only really apparent when uptake was blocked with PDC. In my studies, it would have been interesting to see whether CNQX further reduced the increase in GABA release in PDC, which would also support the possibility of spillover of glutamate release to the GABA terminals. There is clearly a precedent for this at GABA synapses on CA1 interneurones (Kullman and Semyanov, 2002).

Thus, reduction in $[Mg^{2+}]_o$ causes an increase in frequency of excitatory and inhibitory spontaneous activity and increased amplitude of evoked responses, which are largely blocked by the presence of the GluR5-selective antagonist UBP 302. This provides support for the suggestion that GluR5-contain KAr are responsible for the induction and/or maintenance of SWO in the EC (Cunningham *et al.*, 2006b). The latter studies suggested that there was an increased GluR5 KAr component to sEPSPs in low Mg^{2+}. This was not seen in the sEPSCs recorded in my studies. However, there was an increase in amplitudes of the postsynaptic KAr eEPSC, which was blocked by UBP 302. Whilst my data point primarily to increased presynaptic GluR5 activity, I cannot rule out increased activation of postsynaptic GluR5-containing receptors at this stage.

It is clear from the intracellular recordings of SWO that the upstate phase is associated with greatly increased excitatory and inhibitory activity (Cunningham *et al.*, 2006b; Haider *et al.*, 2006; Sanchez-Vives and McCormick, 2000). As the SWO can be blocked by UBP 302, this strongly suggests that the GluR5 driven effects on excitatory and inhibitory elements of the network are involved in SWO generation. It is not possible to speculate on the functional mechanisms of network interaction leading to SWO generation from my data. However, they do affirm a primary role of enhanced GluR5 activity. Finally, as noted in the Introduction (Section 6.1), 2-AP5 may also prevent SWO generation. It is interesting that 1, NMDAr and GluR5 KAr may interact cooperatively in mediating autoreceptor facilitation in layer III (Section 4.3.1.6.), and 2, that 2-AP5 may at least partly reduce the enhanced glutamatergic transmission seen in low Mg^{2+}. Thus, interaction between these two presynaptic receptors may also be involved in initiation or maintenance of SWO.

CHAPTER 7

PRESYNAPTIC KAR AT GLUTAMATERGIC SYNAPSES IN LAYER V.

7.1. Introduction

The experiments detailed in the preceding Chapters of this thesis have been conducted in layer III of the EC (for reasons outlined in the Introduction). However, there is little information available from expression studies to define the laminar distribution of KAr subunits in the EC. Postsynaptic KAr-mediated EPSCs have been identified in layer II (West *et al*., 2007). But nothing is known about any possible role of KAr presynaptically in layer II and either pre- or postsynaptically in other layers.

However, in terms of laminar related actions of KAr it is very interesting that the power of the gamma oscillations induced by KA in EC studies shows a peak in layer III which declines sharply in layer V and also towards layer II, suggesting that KAr in layer III are important for the generation of gamma oscillations (Cunningham *et al*., 2003). Also, SWO oscillations in the EC are prominent in layer III (and II) but decline markedly in layer V (Cunningham *et al*., 2006b). Again, as these oscillations can be blocked by UBP 302 they are likely to be driven in part by GluR5 KAr particularly in layer III. Thus, there would appear to be a correlation between KAr activation and the presence of strong oscillations in this layer and this has been a major driving force behind the studies in this thesis.

Having determined a role for KAr both pre- and postsynaptically in layer III, I now asked whether these receptors may be functional in transmission in layer V. Since, both gamma and SWO are much less pronounced in the deep layer and KAr appear to play a dominant role in their generation my starting hypothesis was that KAr may play less of a role here. Thus, in this Chapter I have performed preliminary experiments to examine the role of KAr in layer V. To date, these have been aimed at examining a possible presynaptic role at glutamate synapses.

7.2. Methods

Again, brain slices were prepared from juvenile animals aged from 4 to 6 weeks old (45 - 70 g). sEPSCs, mEPSCs and eEPSCs were recorded from neurones in layer V of the medial division of the EC.

No specific analysis of the type of neurone recorded was performed. However, visual observation showed that the majority of neurones were medium-sized pyramidal neurones. Recordings were essentially limited to neurones with this morphology, with the presumption that these were principal glutamatergic neurones.

Frequency-dependent facilitation of AMPAr-mediated eEPSCs is a prominent feature of excitatory transmission in layer V but this is heavily dependent on presynaptic NMDAr activation (Woodhall *et al*., 2001a; Yang *et al*., 2006a). In order to compare the involvement of presynaptic NMDAr in layer V with a possible role of presynaptic KAr the protocol for evoking eEPSCs and repetitive stimulation was slightly modified compared to that used in previous Chapters and designed to match that used in layer V previously in this laboratory. Single stimuli were delivered continuously at 0.05 Hz. At 2 minute intervals these were replaced briefly with trains of stimuli (3 Hz, 5 s) before low frequency (0.05 Hz) stimulation was resumed. Frequency-dependent facilitation was quantified by averaging the largest 5 responses evoked during the train and comparing these to the amplitude of 5 low frequency responses directly before each train. Mean facilitation in a minimum of 3 trains was then compared in the presence and absence of the drugs.

In all the experiments, MK 801 was included in the patch pipette solution. This has been shown to block the postsynaptic NMDAr on the recorded neurone (Berretta and Jones, 1996b; Woodhall *et al*., 2001a; Yang *et al*., 2006a) allowing examination of presynaptic NMDAr-mediated effects in isolation. I used this approach to ensure consistency in my comparisons between NMDAr and KAr actions.

7.3. Results

7.3.1. ATPA has no effect on frequency of mEPSCs in layer V

I showed in Chapter 4 (Section 4.3.1.1.) that ATPA powerfully increased the frequency but not amplitude of sEPSCs and mEPSCs in layer III neurones, providing strong evidence for presynaptic KAr autoreceptors on glutamate terminals. I have now conducted similar experiments in layer V neurones. I restricted these experiments to examining mEPSCs as I wished to determine if KAr were present specifically on

terminals on layer V neurones without complication from network/activity driven effects on glutamate release.

mEPSCs were recorded in layer V neurones in the presence of TTX (1 µM). In the presence of ATPA (0.5 µM) there was no discernible change in mEPSCs. The IEI in ATPA was 702 ± 37 ms compared to 652 ± 73 ms in control. The cumulative probability analysis of IEI showed completely overlapping distributions in the pooled data (Figure 7.1A). In addition, ATPA failed to alter the amplitude (11.8 ± 0.4 pA compared to 11.3 ± 1.4 pA in control), time to decay (7.0 ± 1.3 ms compared to 5.7 ± 0.8 ms in control; Figure 7.1B) or 10-90% rise time (1.4 ± 0.3 ms compared to 1.1 ± 0.2 ms in control). Thus, these data indicate that GluR5 receptors are not present on glutamate terminals in layer V, in contrast to layer III (Chapter 4).

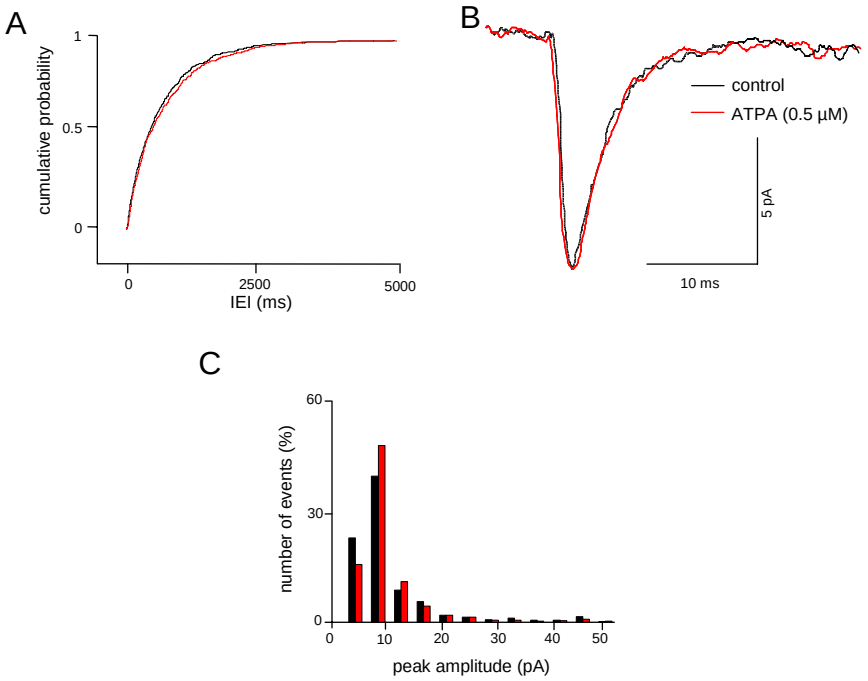


Figure 7.1. Effects of ATPA on mEPSCs in layer V of the EC. *A: Cumulative probability graph of mEPSC IEI in 3 neurones in control conditions and after application of ATPA (0.5 µM). B: Average mEPSC (n=60) in control and in the presence of ATPA. C: Graph of amplitude distribution for pooled data (n=3) in control conditions and in the presence of ATPA (bin size = 4).*

7.3.2. 2-AP5 blocks facilitation of glutamate release in layer V.

Frequency-dependent facilitation of evoked glutamatergic responses has been shown to be a pronounced feature of excitatory transmission in layer V (Woodhall *et al.*, 2001a; Yang *et al.*, 2006a). This occurs at frequencies as low as 2-3 Hz, and is

strongly dependent on presynaptic NMDAr activation (Woodhall *et al*., 2001a; Yang *et al*., 2006a; Yang *et al*., 2008). I repeated the protocol used by Yang *et al*. (2006a). MK801 was included in the patch solution in all experiments.

In 4 neurones, average amplitude of the eEPSC during 0.05 Hz stimulation was 128.8 ± 43.6 pA. During the higher frequency (3 Hz) stimulation this increased significantly to 223.9 ± 61.5 pA (P<0.05, n=4; see Figure 7.2A), representing an average increase of 86 ± 27 %. In confirmation of previous studies (Woodhall *et al*., 2001a; Yang *et al*., 2006a), addition of 2-AP5 (50 μM) had no effect on the amplitude of the eEPSC at 0.05 Hz stimulation (138.8 ± 48.0 pA) but significantly decreased the amplitude of the eEPSC at 3 Hz stimulation to 139.9 ± 47.8 pA (P<0.05; n=4; Figure 7.2A) reflecting a decrease in percentage facilitation to 2 ± 3 %.

sEPSCs were also analysed in the same 4 neurones. IEI increased in the presence of 2-AP5 from 315 ± 90 ms to 455 ± 96 ms. This represented a decrease in frequency from 4.0 ± 1.0 Hz to 2.7 ± 0.9 Hz. There was also no change in amplitude or kinetics of the sEPSCs in 2-AP5 (Table 7.1). Thus, 2-AP5 reduced frequency facilitation of eEPSCs and increased IEI of sEPSCs, confirming the tonic facilitatory role of presynaptic NMDAr in layer V (Woodhall *et al*., 2001a).

7.3.3. UBP 302 has no effect on glutamate release in layer V

I now examined whether there is any contribution of presynaptic GluR5 KARs to facilitation of glutamate release in layer V, as is the case in layer III. This protocol, as above, was repeated in the presence of UBP 302 in 5 neurones. Again in control there was a significant frequency-dependent facilitation of eEPSCs. Mean amplitude of eEPSCs was 79.8 ± 6.5 pA in 0.05 Hz stimulation compared to 181.8 ± 14.9 pA in 3 Hz. This represented a mean facilitation of 128 ± 3 %. Addition of UBP 302 (20 μM) had no significant effect on the mean amplitude of the eEPSC either during low frequency stimulation (95.8 ± 10.8 pA) or during high frequency stimulation (203.2 ± 21.4 pA), representing an increase in facilitation of 113 ± 6 %. The increase was significant (P<0.01, n=5; Figure 7.2) and was unchanged when compared with control. sEPSCs were also analysed in these four neurones. There were no significant changes, amplitude or kinetics of the events (Table 7.1). Most importantly there was

no significant change in IEI. If anything there was a very weak decrease, but no overall change in frequency.

sEPSCs	IEI (ms)	Amplitude (pA)	Decay time (ms)	Rise Time (ms)
control	315 ± 90	16.0 ± 2.8	4.7 ± 1.0	1.7 ± 0.4
+ 2-AP5	455 ± 96	15.2 ± 3.0	5.3 ± 1.5	1.7 ± 0.4
control	658 ± 165	15.2 ± 2.6	5.8 ± 1.2	1.8 ± 0.3
+ UBP 302	581 ± 194	16.0 ± 3.1	5.9 ± 0.3	1.6 ± 0.2

Table 7.1. IEI, amplitude, decay and rise times for sEPSCs in layer V.

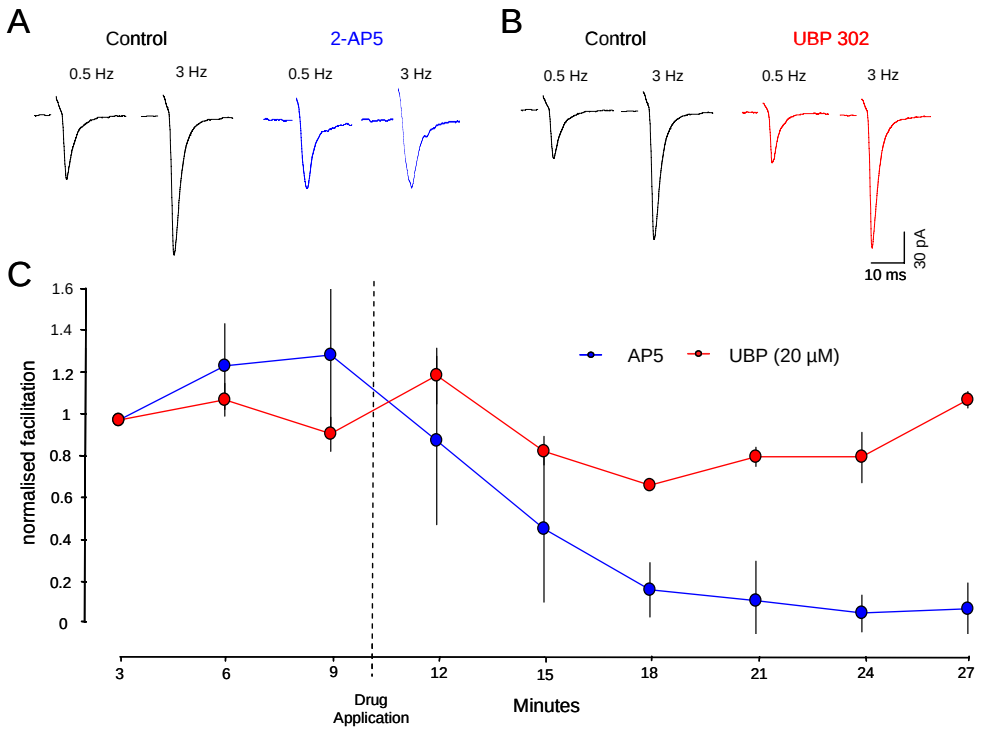


Figure 7.2. Effects of 2-AP5 and UBP 302 on frequency facilitation in layer V. *A: Average eEPSCs (n=6) during 0.5 Hz and 3 Hz stimulation in control conditions and after application of 2-AP5 (50 µM). B: Average eEPSCs (n=6) during 0.5 Hz and 3 Hz stimulation in control conditions and after application of UBP 302 (20 µM). C: Graph of average facilitation from pooled data (n=5) at various time points before and after application of 2-AP5 (red) and UBP 302 (blue.)*

7.3.4. 2-AP5 vs. UBP 302 in layer III

The data above show that facilitation of glutamate release is NMDAr dependent in layer V, but does not involve GluR5 receptors, at least at 3 Hz. The experiments in Sections 4.3.1.4. and 4.3.1.5., examining GluR5-mediated facilitation in layer III,

were all conducted using a frequency of 5 Hz or higher. For completeness, and to directly compare facilitation between layers III and V, I returned to layer III and repeated the experiments using the same protocol as I used in layer V.

In 3 neurones, mean amplitude of the eEPSC increased from 77.6 ± 11.0 pA during 0.05 Hz stimulation. Using this protocol, similar levels of facilitation were found in layer III neurones, with a mean amplitude of 154.3 ± 5.5 pA during high frequency (3 Hz) stimulation (P<0.05, n=3; Figure 7.3). This represents an average increase of 109 ± 27 %. To examine the contribution of presynaptic NMDAr to the facilitation in layer III, we then added 2-AP5 (50 µM) to the bath. The mean amplitude of the eEPSC during 0.05 Hz stimulation was not significantly increased (87.6 ± 13.2 pA). In addition, during high frequency (3 Hz) stimulation mean amplitude remained in the presence of 2-AP5 (mean amplitude of 169.7 ± 17.1 pA; P<0.01, n=3; Figure 7.3). Therefore, there was no significant change in the average percentage facilitation when compared with control (to 99 ± 13 %). These data indicate that facilitation at 3 Hz in layer III does not involve post- or presynaptic NMDAr as facilitation is maintained with MK-801 (1mM) present in the patch pipette and after application of 2-AP5 (50 µM). It should be noted here that experiments in Section 4.3.1.6. reported that 2-AP5 did block facilitation at 5 Hz in layer III. Whether this is due to a difference in stimulation protocols remains to be determined. However, the major difference between the studies was that in the present ones, MK-801 was present in the patch pipette. This may indicate that postsynaptic NMDAr are involved in the facilitation noted earlier (Sections 4.3.1.4, 5 and 6) and that this acts cooperatively with presynaptic GluR5 KAr. This will be a very interesting avenue for future research.

In a further 4 neurones, I repeated the previous experiments but used UBP 302 instead of 2-AP5. In control there was a significant facilitation of eEPSCs, from 81.8 ± 19.3 pA in 0.05 Hz stimulation to 160.7 ± 41.0 pA in 3 Hz (P<0.05; n=4). This represented a mean facilitation of 102 ± 26 %. Addition of UBP 302 (20 µM) had no significant effect on the average amplitude of the eEPSC during low frequency stimulation (119.7 ± 30.47 pA). However, during high frequency stimulation the mean amplitude of the eEPSCs decreased to 129.4 ± 29.7 pA, representing a clear decrease in percentage facilitation to 10 ± 7 % (P<0.05; n=3). These results indicate that UBP 302 but not 2-AP5 removes facilitation at 3 Hz in layer III, suggesting a predominant facilitatory role of GluR5 over NMDAr at excitatory synapses in layer III.

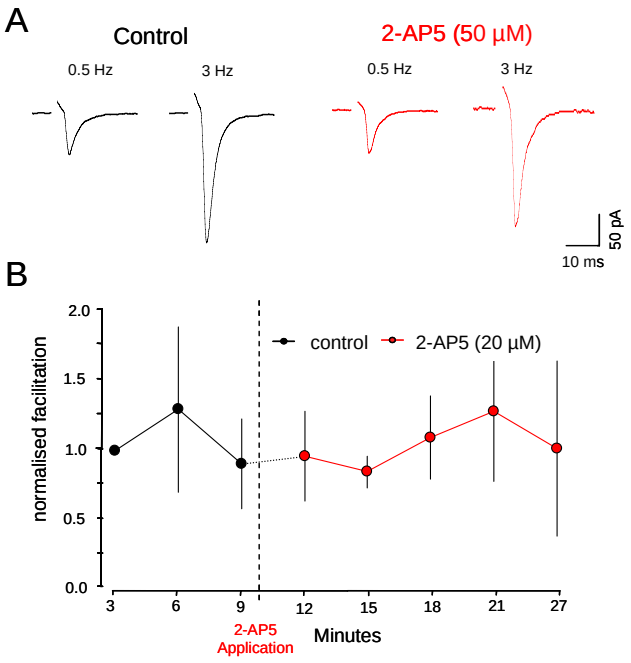


Figure 7.3. Effects of 2-AP5 on frequency facilitation in layer III. *A: Average eEPSC (n=6) in 0.5 Hz and 3 Hz stimulation in control conditions and after 2-AP5 (50 µM) application. C: Graph of average facilitation (n=3) before and after 2-AP5 application.*

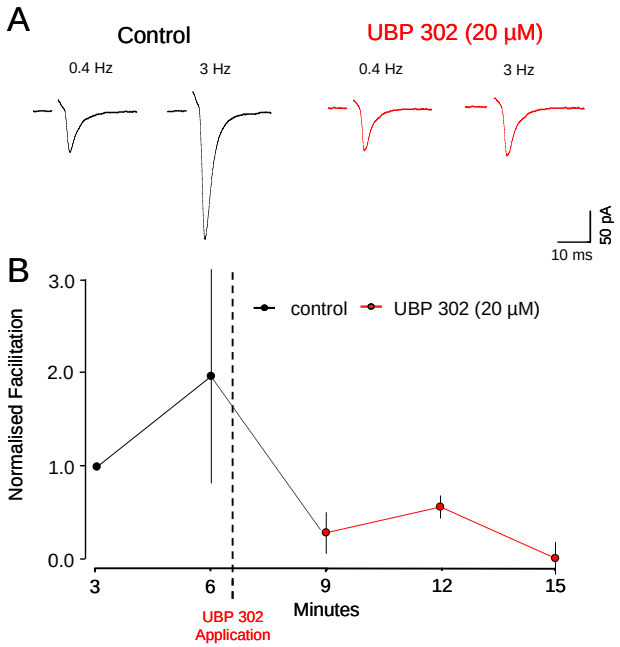


Figure 7.4. Effects of UBP 302 on frequency facilitation in layer III. *A: Average eEPSC (n=6) in 0.5 Hz and 3 Hz stimulation in control conditions and after UBP 302 (20 µM) application. C: Graph of average facilitation (n=3) at various time points before and after UBP 302 application.*

7.3.5. KA decreases inter-event interval of miniature EPSCs in layer V

Thus, the experiments above strongly suggest that GluR5 receptors do not act as autoreceptors in layer V. However, they do not rule out any involvement of KAr containing other subunits. In preliminary studies to look at this possibility I determined the effects of a low concentration of KA itself on mEPSCs recorded in the presence of TTX. KA, at this concentration should non-selectively activate any KAr present on terminals. Application of KA (300 nM) caused a decrease in frequency of mEPSCs in 3 layer V neurones from 3.5 ± 3.3 Hz to 2.2 ± 2.4 Hz, reflecting a change in IEI from 511 ± 229 ms to 900 ± 407 ms. Although the change was not pronounced, KS analysis of cumulative probability distribution showed that it just reached significance (P<0.05; n=3; Figure 7.5.). There was no significant change in mean amplitude (from 11.21 ± 0.93 pA to 12.10 ± 1.07 pA), although there was a tendency towards increase. Likewise, there were no significant changes in kinetics (Figure 7.5B; Table 7.2).

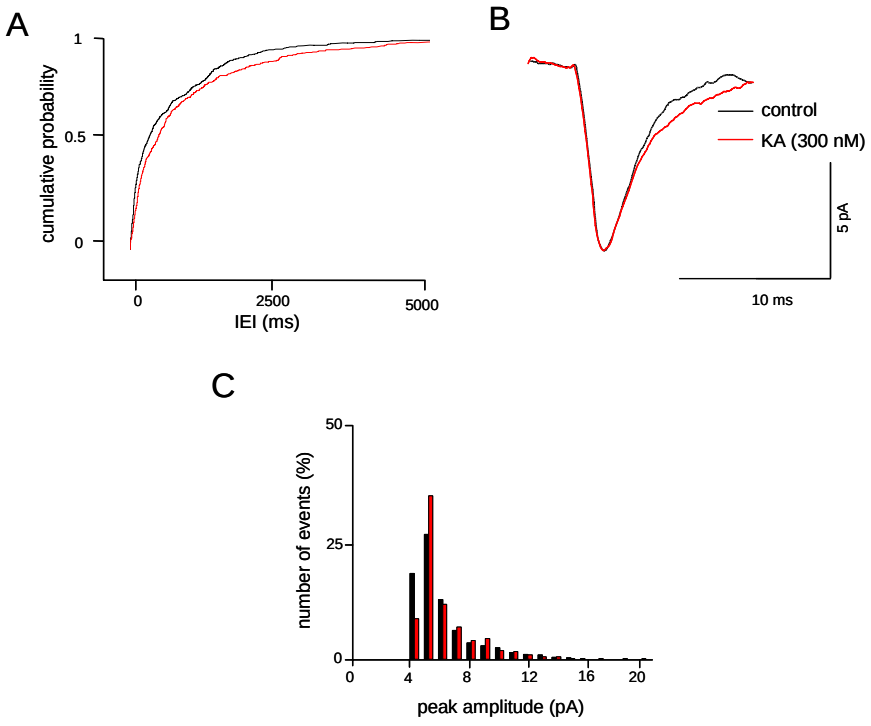


Figure 7.5. Effects of KA on mEPSCs in layer V of the EC. *A: Cumulative probability graph of IEI of mEPSCs in 3 neurones in control conditions and after application of KA (300 nM). B: Average mEPSC (n=60) in control and in the presence of KA (300 nM). C: Graph of amplitude distribution for pooled data (n=3) in control conditions and in the presence of KA (bin size = 3).*

mEPSCs	IEI (ms)	Amplitude (pA)	Decay time (ms)	Rise Time (ms)
control	511 ± 229	11.2 ± 0.9	6.0 ± 1.4	1.1 ± 0.2
+ KA	900 ± 407*	12.1 ± 1.1	7.8 ± 1.8	1.3 ± 0.2

Table 7.2. IEI, amplitude and kinetics for mEPSCs in control and KA in layer V. * *indicates significant change when compared to control (*P < *0.01 KS Test).*

Thus, in complete contrast to layer III excitatory or inhibitory synapses, these studies provide an indication for a KAr that depresses release in layer V.

7.4. Discussion

In Chapter 4 I provided evidence for the presence of presynaptic GluR5 containing (both homomeric and heteromeric) KAr at excitatory synapses in layer III of the EC, where they act to facilitate glutamate release. In addition, in Chapter 6 we have shown that reducing extracellular magnesium from 2 mM to 1.25 mM led to an increase in glutamate release resulting in tonic activation of these presynaptic KAr. This may contribute to the apparent role of GluR5 containing KAr in SWO in layer III of the EC (Cunningham *et al*., 2006b).

In contrast, I have now shown that there is little evidence for the presence of GluR5 containing KAr at excitatory synapses in layer V, as the GluR5 selective agonist ATPA has no effect on the frequency of mEPSCs, and UBP 302 failed to alter frequency-dependent facilitation in this layer. However, the addition of a submicromolar concentration of KA, which is sufficiently low to assume little or no activation of AMPAr, altered the frequency of mEPSCs in layer V. This strongly suggests that there may be a presynaptic KAr present in this layer but in contrast to layer III, these receptors do not contain the GluR5 subunit. Also in marked contrast to layer III, application of KA decreased mEPSC frequency, so it seems likely that these presynaptic KAr act to depress glutamate release from these terminals. Similar depressant effects have been reported in the nucleus accumbens (Casassus and Mulle, 2002), in the barrel cortex (Kidd *et al*., 2002) and in the dorsal horn of the spinal cord (Kerchner *et al*., 2001). Unfortunately without a specific antagonist it is difficult to investigate whether this receptor is tonically active at physiological conditions, like the presynaptic facilitatory NMDAr in this layer (Berretta and Jones, 1996; Woodhall

et al., 2001a). It is also impossible to determine what the relationship between the presynaptic NMDAr and a depressant KAr may be in the overall balance of activity-dependent control of synaptic transmission.

This crucial difference between the function of KAr in layers III and V may underlie some of the fundamental differences between these layers. Thus, whereas SWO are spontaneously generated in layer III by reducing $[Mg^{2+}]_o$, SWO appear virtually absent in layer V (Cunningham *et al*., 2006b). Likewise, gamma frequency oscillations, driven by KA or ATPA are much more pronounced in layer III compared to layer V (Cunningham *et al*., 2003). Indeed, it could be suggested that the depressant KAr that seems to be present in layer V could actively contribute to suppression of SWO and/or gamma in layer V. Obviously much more work is now required to determine the overall contribution of KAr to transmission in layer V, particularly with respect to postsynaptic effects and control of GABA inhibition.

In Chapter 4 (Section 4.4.), I discussed how the facilitatory KAr might be considered a liability, especially if there is no provision for bidirectional modulation of transmitter release. A relatively small increase in glutamate release could lead to a profound and self sustaining excitation. Thus, it may be the presence of this receptor in layer III which underlies the susceptibility to neuronal death seen in TLE. The fact that this type of KAr is not present in layer V may lend support to this argument as the neuronal death in both patients and animal models is largely restricted to layer III and is much less pronounced in the deeper layers (Du *et al*., 1993; Du *et al*., 1995).

It is clear that autoreceptor control of glutamate release is mediated by different mechanisms in neurones which provide hippocampal input (GluR5, layer III) and those that largely relay output (NMDAr, layer V). The functional significance of these differences is obviously unknown at present. However, it is interesting that facilitation is tonically active in layer V but not in layer III. It has been speculated, that layer V neurones represent an “excitable” or “seizure-sensitive” population, whereas the more superficial layers may be more “quiescent” or “seizure resistant” (Jones, 1993; Jones and Lambert, 1990). The differences in autoreceptor control could be a factor in this difference.

CHAPTER 8

GENERAL DISCUSSION

The aims of this thesis were to determine the role of KAr in layer III of the EC using whole-cell patch recordings in rat brain slices.

8.1. Excitation and inhibition in layer III.

Neurones in layer III exhibited continuous spontaneous excitatory and inhibitory activity and the characteristics of sEPSCs and sIPSCs were generally similar to those recorded in the other layers of the EC (Berretta and Jones, 1996a; Woodhall *et al*., 2001; Jones and Woodhall, 2005; Woodhall *et al*., 2005). sEPSCs were abolished by NBQX (and other AMPAr antagonists) and therefore are largely mediated by non-NMDAr, primarily AMPAr. sIPSCs were completely abolished by gabazine, and therefore are mediated by $GABA_A$ receptors but there was effect on holding current indicating an absence of a tonic background current that is a feature of GABAergic synapses in other brain regions (Brickley *et al*., 1996; Nusser and Mody, 2002). In addition, there may be some contribution of glycine receptors in layer III, as strychnine slightly reduced frequency. This was not seen in layers II and V (Woodhall *et al*., 2005). However the effect of strychnine was small, and therefore any contribution of glycine receptors to total background inhibition is unlikely to be significant.

More in depth analysis revealed some differences between layer III and layers II and V. What is perhaps surprising is that there are substantial differences between layer III and layer II, which provide the afferent input to the hippocampus, and substantial similarities to layer V, which is the major relay of hippocampal output. The most pronounced difference between the sEPSCs in layer III compared the other layers is the much higher frequency of events. This may be a reflection of a high baseline firing rate in glutamatergic principal neurones via extensive recurrent excitatory collaterals. This was supported by the fact that a large majority of sEPSCs in layer III were action potential-dependent, as TTX decreased frequency of events by more than six times. There was also a very high level of action potential driven inhibition in layer III, Taken together with the high level of activity driven excitation, may indicate a high degree of interconnectivity between principal neurones and between principal neurones and interneurones in this layer. The pronounced burst activity in both excitatory and inhibitory activity may also reflect this interconnectivity. Currently

missing from this picture is an examination of spontaneous activity in the interneurones themselves, and future experiments should examine this.

8.2. KAr at glutamatergic synapses

Chapter 4 provided electrophysiological evidence for the presence of two different types of KAr involved in glutamatergic neurotransmission in layer III of the EC. Frequency-dependent facilitation of eEPSCs at excitatory synapses appeared to be mediated by a presynaptic KAr containing the GluR5 subunit, but the lack of effect of UBP 302 on sEPSCs indicated that these receptors are not tonically activated by spontaneous glutamate release, only becoming activated in situations where higher levels of glutamate are present. The studies on eEPSCs provide evidence that the presynaptic KAr is activated during repetitive stimulation and therefore act to further boost excitation. The precise mechanism for the facilitation is currently unknown, although, it seems likely to be ionotropic. Surprisingly, 2-AP5 also abolished frequency facilitation. Thus, it is possible that presynaptic KAr and NMDAr are acting synergistically on some terminals to facilitate glutamate release in layer III. One possibility is that repetitive stimulation activates the presynaptic KAr causing the depolarisation of the terminal, which then results in parallel activation of the presynaptic NMDAr by relieving Mg^{2+} block. However, exogenous activation of GluR5 containing receptors with ATPA suggested that the KA autoreceptor is also capable of causing facilitation without synergy with the NMDA receptor.

In addition to identifying the facilitatory presynaptic GluR5 KAr, I also found that KAr can mediate postsynaptic effects in the EC. The KAr-mediated eEPSC I identified was similar to that previously reported by West *et al*. (2007). Most importantly, time to decay, which is often used to separate KAr-mediated currents from much faster AMPAr-mediated currents, were comparable. The I-V curve for KAr eEPSC in my studies showed significant inward rectification, which is characteristic of KAr containing unedited GluR5 and GluR6 subunits (Bowie & Mayer, 1995; Kamboj *et al*., 1995). These are relatively impermeable to Ca^{2+}. However, although the KAr eEPSC was blocked by CNQX (see also West *et al*., 2007) it was unaffected by UBP 302. Therefore, my data indicate that the postsynaptic KAr in layer III are unlikely to contain GluR5 subunits, but instead may comprise an unedited GluR6 subunit. Elucidation of the exact subunit composition of the

postsynaptic KAr at layer III excitatory synapses awaits the development of more selective subunit antagonists.

Thus, KAr can directly mediate postsynaptic excitation at glutamate synapses, and also boost transmission by presynaptic facilitation. These effects, mediated by different subtypes both required repetitive activation, and thus support a role for both pre- and postsynaptic KAr as boosters or amplifiers of synaptic excitation in response to elevated afferent activity.

8.3. KAr at GABAergic synapses

In Chapter 5 I provided evidence for a role of KAr in enhancing inhibitory transmission in layer III. Again this appeared to occur at two levels. A presynaptic KAr located on the inhibitory terminals facilitated spontaneous GABA release. My pharmacological data suggested that in contrast to glutamate synapses, this was not GluR5 containing but may contain the GluR6/KA2 subunits. Studies with Cd^{2+} suggested that activation of VGCCs must be involved in the mechanism of this facilitation. However, I cannot rule out the possibility that the receptor is also acting via a metabotropic pathway, as has been suggested previously (Rodriguez-Moreno and Lerma, 1998; Braga *et al*., 2003).

Interestingly, UBP 302 caused a decrease in frequency of sIPSCs but not mIPSCs and this indicates that there is a second mechanism of KAr mediated facilitation of GABA release. This seems likely to be a GluR5 containing KAr located on the soma/dendrites of the interneurones, as has also been reported in the hippocampus (Cossart *et al*., 1998; Frerking *et al*., 1998). However, in order to confirm this I would need to record from the interneurones directly. Experiments with eIPSCs support this suggestion, because UBP 302 depressed the amplitude of the first response in the stimulus train and shifted the PPR from PPD to PPF. This PPF is blocked by concentrations of CNQX selective for KAr, supporting the suggestion that there is a presynaptic KAr on the inhibitory terminals facilitating GABA release that may not contain the GluR5 subunit. Thus, there is more than one type of KAr involved in increasing inhibition in layer III, presynaptic GluR6/KA2 receptors and also GluR5 containing KAr driving interneuronal firing.

8.4. Reduction of $[Mg^{2+}]_o$ – roles of KAr in SWO

SWO can be generated in the EC by reducing $[Mg^{2+}]_o$ from 2 mM to 1.25 mM and work by Cunningham *et al*. (2006b) indicated that KAr containing the GluR5 subunit are involved in the initiation and/or maintenance of these SWO.

In Chapter 6 I showed that the same paradigm of reducing $[Mg^{2+}]_o$ caused an increase in sEPSCs and an increase in amplitude of the eEPSCs (both those mediated by AMPAr and KAr). Increased activation of presynaptic or postsynaptic NMDAr was not responsible for the increased release observed in reduced Mg^{2+}, and pharmacological experiments showed that a large part of the increased release was due to increased activation of the GluR5 KA autoreceptor. In low Mg^{2+} these GluR5 receptors were tonically activated by ambient glutamate. Overall, my data suggest that glutamate release may be increased generally (probably by reduced blockade of VGCC by Mg^{2+}) and this increased release is then boosted by enhanced activation of the presynaptic GluR5 receptors. This was supported by the evidence that blocking glutamate uptake had virtually identical effects to reducing $[Mg^{2+}]_o$. At present, I cannot completely rule out the possibility that PDC and low Mg^{2+} increase glutamate release, which can then access postsynaptic GluR5 KAr not normally activated by ambient or evoked glutamate release. Both manipulations caused an increase in the KAr eEPSC and PDC also slightly increased sEPSC amplitude, these effects were partially reversed by UBP 302. However, the studies in 2 mM $[Mg^{2+}]_o$ (Section 4.3.1.) strongly indicated a presynaptic GluR5 KAr and low Mg^{2+} increases sEPSC frequency with no effect on amplitudes or kinetics. Therefore, overall my conclusion is that the effects are almost certainly due to increased presynaptic GluR5 activity.

Reduction of Mg^{2+} in the aCSF also caused a pronounced increase in sIPSC frequency and an increase in amplitude of the eIPSCs. eIPSCs were also increased in amplitude although frequency-dependent depression was unaffected. Again, it seems likely that increased GABA release will occur at least partly due to removal of a Mg^{2+} block on the presynaptic VGCC on the GABAergic terminal. However, UBP 302 and CNQX were both able to reduce the effects of low Mg^{2+} in a way which supported the earlier conclusions regarding the role of KAr in control of inhibition. Thus, firstly, antagonist studies were consistent with a contribution of increased activation of GluR5 containing KAr as a result of increased glutamate release at excitatory synapses on the

interneurones. Secondly, they suggested that there was increased activation of GluR6/KA2 receptors on the GABA terminals, again as a result of increased glutamate spillover. Studies with PDC accorded well with these effects being partly due to increased glutamate levels present at KAr on the interneurones and their terminals.

8.5 KAr in layer V of the EC

Oscillatory activity involving KAr is much less pronounced in layer V of the EC compared to layer III (Cunningham *et al*., 2003; 2006b), so in Chapter 7, I attempted to identify a role for KAr at glutamatergic synapses in layer V. In contrast to layer III ATPA had no effect on mEPSCs, and UBP 302 failed to alter frequency-dependent facilitation suggesting that presynaptic GluR5 KAr are unlikely to facilitate glutamate release in layer V. However, KA itself reduced the frequency of mEPSCs in layer V strongly suggesting that there may be a non-GluR5 containing presynaptic KAr acting to depress glutamate release at these synapses. It would be interesting to determine whether this receptor is tonically active and what the relationship between the presynaptic NMDAr in layer V (Berretta and Jones, 1996b; Woodhall *et al*., 2001) and a depressant KAr may be in the overall balance of activity-dependent control of synaptic transmission. Unfortunately, without a specific antagonist it is difficult to investigate this receptor further.

This crucial difference between the function of KAr in layers III and V may underlie some of the fundamental differences between these layers. Thus, whereas SWO are spontaneously generated in layer III by reducing $[Mg^{2+}]_o$, they are virtually absent in layer V (Cunningham *et al*., 2006b). Likewise, gamma frequency oscillations, driven by KA or ATPA are much more pronounced in layer III compared to layer V (Cunningham *et al*., 2003). Indeed, it could be suggested that the depressant KAr that seems to be present in layer V could actively contribute to suppression of SWO and/or gamma in layer V. Obviously much more work is now required to determine the overall contribution of KAr to transmission in layer V, particularly with respect to postsynaptic effects and control of GABA inhibition. However, it is clear that autoreceptor control of glutamate release is mediated by different receptors in neurones which provide hippocampal input (GluR5, layer III) and those that largely

relay output (NMDAr, layer V). The functional significance of these differences is obviously unknown at present.

8.6. Perspectives

As frequently noted, KAr have been shown to have roles in oscillatory activity. KAr have been shown to play a role in gamma oscillations in the hippocampus (Brown *et al*., 2006) and the EC (Cunningham *et al*., 2003), theta oscillations in the hippocampus (Huxter *et al*., 2007) and SWO in the EC (Cunningham *et al*., 2006; Stanger *et al*., 2008).

Is it possible to relate the observations in this thesis to mechanisms of rhythmicity in layer III? Generation of oscillatory activity in cortical networks is likely to depend on the dynamics of network interconnectivity and firing patterns in constituent neurones (see collected papers in "Structure/function correlates in neurones and networks" J. Physiol. 2005: 526(1)). The connectivity in layer III of the EC indicated by analysis of spontaneous activity in my studies, and by previous studies of recurrent excitation (Dhillon and Jones, 2001) could therefore be a factor in the susceptibility of this layer to generation of gamma oscillations and SWO (Cunningham et al., 2003; 2006b). It is also clear that KAr act to increase transmission at both excitatory and inhibitory synapses in layer III. Currently, it is not known whether the different sub-type specific KAr effects are further location-specific in terms of types of interneurones/principal neurones, or pathway specific (e.g. recurrent versus afferent connections, feed-forward or feed back pathways), so it would be premature to speculate on precise mechanisms. However, it is clear that an end result of KAr stimulation can be to induce gamma oscillations in layer III (Cunningham *et al*., 2003b), and that blocking KAr can modify SWO (this thesis; Cunningham *et al*., 2006; Stanger *et al*., 2008). My experiments have begun to provide a framework for delineating site and function-specific role of KAr in these phenomena.

Rhythmic or synchronous electrical activity at various frequencies is seen all over the CNS and is thought to be important in many processes including memory (for review: see Axmacher *et al*., 2006). Gamma oscillations have been suggested to be responsible for long-term storage of consciously accessible information into memory (Fell *et al*., 2001, Gruber *et al*., 2004 and Sederberg *et al*., 2003) and lesion studies

have demonstrated that structures within the medial temporal lobe are necessary for this memory formation (e.g., Scoville and Milner, 1957). SWO, which are particularly prominent during slow wave sleep, are suggested to be important in the transfer of memory traces from the hippocampus to the neocortex during sleep (for review see Hoffman *et al*., 2007). If SWO are important for learning and memory, for underlying the transfer of memory traces from the hippocampus to the neocortex during sleep as has been suggested (Destexhe *et al*. 2007) and KAr are important in the generation of SWO in the EC, then changes in KAr function in the EC could be responsible for some of the deficits in memory that are seen in various diseases. There is some evidence for this. Di Paola *et al*. (2007) found that EC atrophy in Alzheimer's patients was correlated with a decline in performance of episodic memory delayed recall tests. Takeda *et al*. (2007) found that, in postmortem studies, a number of amyotrophic lateral sclerosis patients with signs of dementia showed degeneration of the perforant pathway, and that this degeneration was more pronounced in patients who showed severe memory deficits. In addition, significant reductions in the size of the EC have been found in patients with both subjective memory and mild cognitive impairments (Jessen *et al*., 2006). There is also some evidence to suggest the involvement of GluR5-containing KAr in learning and memory. Barker *et al*. (2006) infused a GluR5-selective antagonist into the perirhinal cortex in rats and found that it produced an impairment in recognition memory that was independent of the impairment produced by an NMDAr antagonist. GluR5 KAr have also been implicated in mossy fibre LTP (Bortolotto *et al*., 1999, 2005).

An inability to integrate or synchronise activity in neuronal networks in increasingly thought to underlie the cognitive symptoms of schizophrenia. KAr have been repeated implicated in aspects of oscillatory activity both in the EC (Cunningham *et al*., 2003, 2006; Stranger *et al*., 2008) and in the hippocampus (Brown *et al*., 2006; Goldin *et al*., 2007; Huxter *et al*., 2007). Disruption of KAr function in schizophrenia may lead to abnormal neuronal synchrony in the EC and, thus, cause a number of these symptoms. There has been some evidence for the involvement of KAr, specifically the GluR5 subunit, in schizophrenia. Woo *et al*. (2007) showed that in post-mortem studies of schizophrenic patients the density of the GAD(67) mRNA-containing neurones that co-expressed GluR5 mRNA was decreased in the anterior cingulate cortex. Scarr *et al*. (2005) have also shown that [3H]KA binding was significantly decreased in the cortex of patients with schizophrenia. By contrast, levels of [3H]MK-801 and [3H]AMPA

binding did not differ, suggesting that there is a change in KAr number but not NMDAr or AMPAr. In addition, GluR5 mRNA is decreased in the cortex of patients with schizophrenia whilst there is difference in levels of mRNA for the other KAr subunits (Scarr *et al*., 2005). In other post-mortem studies, a reduction of GluR5/6/7 immunoreactivity has been observed on apical dendrites of hippocampal pyramidal neurones in schizophrenic brains (Benes *et al*., 2001).

Changes in the ability to maintain normal network synchrony will also have obvious consequences in epilepsy. GluR5-containing KAr function has also been shown to be altered in epilepsy. GluR5 and GluR6 KAr mRNA has been shown to be reduced in hippocampal pyramidal cells post-mortem studies of TLE patients with hippocampal sclerosis (Mathern *et al*., 1998). Concurrently there was an increase in GluR5 and KA2 mRNA in dentate granule cells (Mathern *et al*., 1998). In addition, the allelic varients of GluR5 but not GluR6 confers genetic susceptibility to the development of juvenile absence seizures (Sander *et al*., 1997; Sander *et al*., 1995 respectively). It is possible that changes in KAr function in the EC may be involved in TLE, as the EC has repeatedly been implicated in TLE (Rutecki *et al*., 1989; Spencer and Spencer, 1994; Alarcon *et al*., 1997; Assaf and Ebersole, 1997; Bernasconi *et al*., 1999, 2001; Jutila *et al*., 2001; Wennberg *et al*., 2002; Asaf *et al*., 2003; Bonilha *et al*., 2003; Bartolomei *et al*., 2004, 2005; Jamali *et al*., 2006).

Finally, it is interesting to note that several studies have suggested that a reduction in CSF Mg^{2+} could be, in part, responsible for the clinical manifestation of several pathological conditions. Uitti *et al*. (1989) showed that there were decreased Mg^{2+} concentrations in the caudate nucleus in parkinsonian brains. In agreement with this, a more recent study has shown that there is decreased free Mg^{2+} in CSF of Parkinson's disease patients (Barbiroli *et al*., 1999). Decreased plasma Mg^{2+} concentrations have also been found in patients with dementia of Alzheimer's type (Lemke, 1995). Interestingly, Mg^{2+} content in specific regions associated with Alzheimer's disease has also been found to be reduced, including in the EC where a 20% reduction was found on average (Andrasi *et al*., 2005). Intracellular Mg^{2+} concentration has been found to be increased in the prefrontal cortex of schizophrenic patients (Hinsberger *et al*., 1997). However, Levine *et al*. (1996) found that acute schizophrenic patients have significantly lower CSF Mg^{2+} concentrations than patients in remission. This study is in agreement with earlier findings that that blood plasma Mg^{2+} levels were decreased

in schizophrenic patients but increased back to control levels when clinical remission was achieved (Kirov and Tsachev, 1990). Finally, the induction of epilepsy by lowering/removing extracellular Mg^{2+} concentration is a widely used *in vitro* model of epilepsy (Jeffreys, 1998) and CSF Mg^{2+} concentration is decreased in children with epilepsy when compared to control (Miyamoto *et al*., 2004). In addition, low CSF Mg^{2+} positively correlated with increased frequency, poor control and longer duration of fits in patients with iodiopathic generalised tonic clonic seizures (Sood *et al*., 1993).

My results have demonstrated several roles for KAr in layer III of the EC and have provided support for the suggestion that they are involved in SWO in this region. These findings could be relevant for the development of therapeutic interventions for pathological conditions where the EC, KAr and disruption in normal network synchrony has shown to be important.

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Mobility of NMDA autoreceptors but not postsynaptic receptors at glutamate synapses in the rat entorhinal cortex

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NMDA receptors (NMDAr) are known to undergo recycling and lateral diffusion in postsynaptic spines and dendrites. However, NMDAr are also present as autoreceptors on glutamate terminals, where they act to facilitate glutamate release, but it is not known whether these receptors are also mobile. We have used functional pharmacological approaches to examine whether NMDA receptors at excitatory synapses in the rat entorhinal cortex are mobile at either postsynaptic sites or in presynaptic terminals. When NMDAr-mediated evoked EPSCs (eEPSCs) were blocked by MK-801, they showed no evidence of recovery when the irreversible blocker was removed, suggesting that postsynaptic NMDAr were relatively stably anchored at these synapses. However, using frequency-dependent facilitation of AMPA receptor (AMPAr)-mediated eEPSCs as a reporter of presynaptic NMDAr activity, we found that when facilitation was blocked with MK-801 there was a rapid (∼30–40 min) anomalous recovery upon removal of the antagonist. This was not observed when global NMDAr blockade was induced by combined perfusion with MK-801 and NMDA. Anomalous recovery was accompanied by an increase in frequency of spontaneous EPSCs, and a variable increase in frequency-facilitation. Following recovery from blockade of presynaptic NMDAr with a competitive antagonist, frequency-dependent facilitation of AMPAr-mediated eEPSCs was also transiently enhanced. Finally, an increase in frequency of miniature EPSCs induced by NMDA was succeeded by a persistent decrease. Our data provide the first evidence for mobility of NMDAr in the presynaptic terminals, and may point to a role of this process in activity-dependent control of glutamate release.

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Synaptic transmission is a dynamic and plastic process, modified by short, intermediate and long-term regulatory mechanisms. Synaptic strength can be modulated by presynaptic receptors, which provide activity-dependent control of transmitter release. Presynaptic ionotropic receptors have received increasing attention recently (see Engelman & Macdermott, 2004 for review). AMPA receptors (AMPAr; Patel & Croucher, 1997; De Paola *et al.* 2003), NMDA receptors (NMDAr; Berretta & Jones, 1996; Li & Han, 2007; Woodhall *et al.* 2001) and kainate receptors (Agrawal & Evans, 1986; Malva *et al.* 1995; Chittajallu *et al.* 1996; Negrete-diaz *et al.* 2006) can all act as autoreceptors on glutamatergic terminals, and as heteroreceptors on GABA terminals (Bureau & Mulle, 1998; Kullmann & Semyanov, 2002; Duguid & Smart, 2004).

At postsynaptic sites, trafficking of membrane receptors is a determinant of moment-to-moment and long-term modulation of synaptic efficacy (see Carroll & Zukin, 2002; Bredt & Nicoll, 2003; Perez-Otano & Ehlers, 2005; Triller & Choquet, 2005). Interest in receptor mobility has been driven by the demonstration that AMPAr are rapidly inserted or deleted at glutamate synapses during long-term potentiation (LTP) and depression (LTD) (see Ehlers, 2000; Malinow & Malenka, 2002; Song & Huganir, 2002; Bredt & Nicoll, 2003; Collingridge *et al.* 2004). NMDAr were originally thought to be stably anchored at the postsynaptic density (PSD; Ehlers, 2000; Scannevin & Huganir, 2000; Bredt & Nicoll, 2003) but it is now apparent that they also undergo regulated trafficking and that this may be intimately involved in long-term synaptic plasticity (Carroll & Zukin, 2002; Nong *et al.* 2004; van Zundert *et al.* 2004; Perez-Otano & Ehlers, 2005)

The focus regarding glutamate receptor mobility has been on recycling of receptors between the synaptic membrane and intracellular pools. However, diffusion

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of receptors within cell membranes may also regulate synaptic efficacy. Glycine (Meier *et al.* 2001; Dahan *et al.* 2003), $GABA_A$ (Jacob *et al.* 2005; Thomas *et al.* 2005), AMPAr (Borgdorff & Choquet, 2002; Groc *et al.* 2004) and NMDAr (Groc *et al.* 2004) have all been shown to undergo lateral diffusion in the postsynaptic membrane primarily using direct imaging and optical tracking. Lateral diffusion of postsynaptic NMDAr has been demonstrated using a pharmacological approach (Tovar & Westbrook, 2002; Zhao *et al.* 2008), in which irreversible block of NMDAr responses by MK-801 at synapses on hippocampal neurones was followed by an anomalous recovery during washout of the blocker. This suggested that receptors that were use-dependently blocked following activation by glutamate at the PSD could be replaced by non-blocked receptors from a distal source. A number of experimental approaches indicated that this replacement occurred by lateral diffusion of receptors from extrasynaptic sites in the postsynaptic membrane rather than by insertion of new receptors from cytosolic stores (Tovar & Westbrook, 2002; Zhao *et al.* 2008).

In the entorhinal cortex (EC) presynaptic NMDAr facilitate spontaneous release at glutamate synapses (Berretta & Jones, 1996; Woodhall *et al.* 2001; Yang *et al.* 2006). These receptors also mediate short-term, frequency-dependent facilitation of excitatory transmission (Berretta & Jones, 1996; Woodhall *et al.* 2001; Sjöström *et al.* 2003; Brasier & Feldman, 2008; Chamberlain *et al.* 2008), and may play a role in LTP; Humeau *et al.* 2003; Samson & Pare, 2005), LTD; (Sjöström *et al.* 2003; Bender *et al.* 2006; Corlew *et al.* 2007), and activity-dependent signalling via astrocytes (Jourdain *et al.* 2007). The role of presynaptic NMDAr in synaptic plasticity could be modulated by mobility of receptors in the terminal membrane. In the present study we used physiological approaches to examine whether pre- or postsynaptic NMDAr undergo lateral diffusion at glutamate synapses in the EC. Some of these results have been published in abstract form (Yang *et al.* 2008).

Methods

Ethical information

Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, European Communities Council Directive 1986 (86/609/EEC) and the University of Bath ethical review document.

Slice preparation

Slices containing EC and hippocampus were prepared from male Wistar rats (3–5 weeks), which were anaesthetized with an I.M. injection of ketamine ($120\ mg\ kg^{-1}$) plus xylazine ($8\ mg\ kg^{-1}$) and decapitated. The brain was rapidly removed and immersed in oxygenated artificial cerebrospinal fluid (aCSF) chilled to 4°C. Slices (350–400 μm) were cut using a Vibroslice, and stored in aCSF bubbled with 95% O_2–5% CO_2, at room temperature. Following recovery for at least 1 h, individual slices were transferred to a recording chamber mounted on the stage of a Zeiss Axioskop FS or an Olympus BX50WI microscope. The chamber was perfused ($2.5\ ml\ min^{-1}$) with oxygenated aCSF (pH 7.4) at 31–33°C. The aCSF contained (in mM): NaCl (126), KCl (3), NaH_2PO_4 (1.25), $NaHCO_3$ (24), $MgSO_4$ (2), $CaCl_2$ (2), and D-glucose (10). Neurones were visualized using differential interference contrast optics and an infrared video camera.

Electrophysiological recordings

Patch pipettes were pulled from borosilicate glass on a Flaming/Brown microelectrode puller. For recording spontaneous (sEPSCs), miniature (mEPSCs) or evoked (eEPSCs) EPSCs, pipettes were filled with a caesium gluconate-based solution containing (in mM): D-gluconate (100), Hepes (40), QX-314 (1), EGTA (0.6), NaCl (4), $MgCl_2$ (5), TEA-Cl (1), ATP-Na (4), GTP-Na (0.3), MK-801 (2). Solutions were adjusted to $290\ mosmol\ l^{-1}$, and to pH 7.25–7.3 with CsOH. Whole-cell voltage clamp recordings (holding potential –60 mV unless otherwise stated) were made from neurones in layer V of the medial division of the EC, using an Axopatch 200B amplifier. Series resistance compensation was not employed, but access resistance (10–30 MΩ) was monitored at regular intervals throughout each recording and cells were discarded from the analysis if it changed by more than ±10%. Liquid junction potentials were estimated using the calculator of pCLAMP 8 software, and compensated for in the holding potentials.

eEPSCs were elicited by electrical stimulation (bipolar pulses, 10–50 V, 0.02 ms duration) via a bipolar tungsten electrode placed on the surface of the slice in layer V of the lateral EC. The stimulation intensity was adjusted to give a submaximal (approx. 60% maximum amplitude) response. Unless otherwise stated, MK-801 was included in the patch pipettes allowing us to record AMPA-mediated responses in isolation and to monitor activity at presynaptic NMDAr uncontaminated by post-synaptic receptor effects, an approach that we (Berretta & Jones, 1996; Woodhall *et al.* 2001; Yang *et al.* 2006, 2007), and others (Sjöström *et al.* 2003; Samson & Pare, 2005; Bender *et al.* 2006; Jourdain *et al.* 2007; Li & Han, 2007; Brasier & Feldman, 2008), have used successfully to block postsynaptic NMDAr in recorded neurones.

Determination of receptor mobility

Postsynaptic. We used an approach described by Tovar & Westbrook (2002) and used by others (Harris & Pettit, 2007; Zhao *et al.* 2008) to monitor postsynaptic NMDAr mobility. Repeated stimulation delivered in the presence of the channel blocker, MK-801 results in progressive block of NMDAr accessed by glutamate released during synaptic activation. As this block is essentially irreversible, recovery of the synaptic response after washout of MK-801 provides a reporter of receptor mobility. A modification of this approach was used here to monitor the mobility of postsynaptic NMDAr in the EC in two series of experiments. Both were conducted with an AMPAr antagonist (NBQX), and a $GABA_A$r antagonist (bicuculline) in the bath. In the first study, MK-801 was *not* included in the patch pipette solution. When whole-cell access was achieved, neurones were held at +40 mV and isolated, long duration NMDAr-mediated EPSCs were evoked at low frequency (0.05 Hz). These vary in their deactivation kinetics from neurone to neurone (see Fig. 1*A*) but can be abolished by NMDAr antagonists (Chamberlain *et al.* 2008). When eEPSC amplitudes were stable, MK-801 (10 μM) was bath applied for a period of 5 min. Four minutes after MK-801 perfusion commenced, stimulation frequency was increased to 5 Hz for 40–50 s, resulting in a rapid decline in eEPSC amplitude to around 0–10% of the amplitude recorded at low frequency. Stimulation frequency was then restored to 0.05 Hz, and MK-801 was washed out of the bath. Responses to low frequency stimulation were then monitored for the remaining lifetime of the recording (e.g. Fig. 2). After 35 min, all stimulation was halted for a period of 15 min, before being resumed at low frequency until the end of the recording.

In a second series of experiments, the same protocol was followed, but MK-801 was omitted from the external perfusion and included in the patch pipette. Again, when whole-cell access was achieved, NMDAr-mediated eEPSCs were recorded at low frequency (0.05 Hz). After 6 min, stimulation frequency was increased to 5 Hz for 40–50 s and this resulted in a rapid decrease of the eEPSCs due to blockade of the postsynaptic NMDAr by intracellular MK-801 dialysed into the cell via the patch pipette. When the eEPSCs had reached a stable low level, stimulation was returned to 0.05 Hz, and recovery of the responses monitored for the lifetime of the patch recordings (Fig. 2).

Presynaptic. Monitoring presynaptic receptor mobility is more difficult. In addition to the electrophysiological approach (Tovar & Westbrook, 2002; Harris & Pettit, 2007), optical tracking of quantum dot or fluorescent antibody-labelled receptors has been used to study postsynaptic receptor trafficking (e.g. Groc *et al.* 2004; Washbourne *et al.* 2004). These approaches are complicated at presynaptic sites because the size of terminals precludes direct electrophysiological access, or sufficient visual resolution. Direct imaging of the movement of receptors in spines and dendrites has been largely conducted in cultured neurones and applying this to terminals in native tissue would be technically more difficult. It requires specific labelling of presynaptic receptors without contamination by those at postsynaptic sites, and a very high degree of visual spatial resolution to visualize and track receptor movement in very small terminals.

To overcome these limitations we have developed a variation of the functional pharmacological approach (Tovar & Westbrook, 2002; Harris & Pettit, 2007) to test for the mobility of presynaptic NMDAr in EC slices. All experiments were conducted with MK-801 in the patch pipette. When whole-cell access was gained, neurones were voltage clamped at 0 mV, and synaptic stimulation delivered at 5 Hz for 30–40 s to allow blockade of postsynaptic NMDAr by MK-801 dialysed into the cell via the patch pipette solution. Membrane potential was then clamped at –60 mV and single shock stimulation delivered at low frequency (0.05 Hz) to evoke isolated AMPAr-mediated EPSCs. At intervals of 2 min, the single shock was replaced with stimulation at 3 Hz for 5 s. Such stimulation results in a frequency-dependent facilitation of the AMPAr-mediated EPSC, which we have shown previously to arise primarily from activation of presynaptic NMDAr (Woodhall *et al.* 2001; Chamberlain *et al.* 2008). We used the degree of frequency-dependent facilitation of AMPAr-mediated eEPSCs as a quantitative measure of presynaptic NMDAr activation. After a control recording of 3–4 episodes of 3 Hz stimulation, with single shock stimulation at 0.05 Hz restored between each episode, MK-801 (10 μM) was bath applied for 5 min and then washed out for the remaining lifetime of the recording (see Figs 3, 4, 5 and 7). The stimulation protocol continued uninterrupted during the period of MK-801 application and during the remainder of the recording. The protocol thus included at least 3–5 periods of stimulation at 3 Hz in the presence of MK-801 (including those occurring when the blocker was being washed from the bath, see Fig. 1*A*), and this was sufficient to cause use-dependent block of frequency-facilitation. In a second group of neurones the stimulation protocol was completely halted for a period of 15 min starting approximately 6 min after the beginning of the MK-801 wash. The rationale for this was to determine if the stimulation, and subsequent evoked release of glutamate, could elicit a use-dependent unbinding of the blocker from the receptors and hence be responsible for recovery. In a third group, the same experimental protocol was employed except that MK-801 perfusion was accompanied by bath perfusion with NMDA (25 μM), and both agents were subsequently washed out. The latter

studies aimed to determine the effects of global blockade of NMDAr on the terminals.

Control studies

MK-801 washout. The majority of the studies involved monitoring response parameters (pre- or postsynaptic) after removal of MK-801 from the bath, and relied on its ability to irreversibly block NMDAr. It was important to attempt to monitor the rate of removal of the drug from the bath after termination of perfusion. The period of application (5 min in all experiments) was timed from the moment the drug solution reached the bath, to the moment it was replaced by drug-free ACSF. The recording chamber had a volume of approximately 500 μl, so with a perfusion rate of 2.5 ml min^{-1} the bath solution should be completely exchanged 4–5 times per minute during washout. Since NMDAr blockade is use dependent, we monitored the washout of MK-801 using the following protocol. Isolated NMDAr-mediated eEPSCs were recorded as above. Control responses were evoked every 15 s for 5–7 min, and then stimulation was halted prior to perfusion with MK-801 for 5 min. Repetitive stimulation (2 Hz for 50 s) was then delivered 4 min into the MK-801 perfusion ($n = 8$), or 2, 7, 12, or 17 min ($n = 3$ in each case) after the start of washout, followed by a return to low frequency stimulation. Responses were averaged over the subsequent 2 min for comparison.

Stability of NMDAr-mediated effects. Experiments on postsynaptic NMDAr mobility rely on the stability of the evoked responses over time. To control for this, in seven neurones we employed the protocol used to monitor postsynaptic mobility (see above) but without the addition of MK-801 to the bath perfusion or the patch pipette. Likewise, meaningful experiments on presynaptic receptor mobility are dependent on frequency-dependent facilitation of AMPAr-mediated EPSCs remaining stable, without rundown over time. To monitor this, in eight neurones we performed experiments designed to monitor presynaptic mobility as described above, but again, without addition of MK-801 to the bath perfusion.

Data analysis

Data were recorded to computer hard disk using Axoscope software. Minianalysis (Synaptosoft, USA) was used for analysis of EPSCs off-line. In experiments with postsynaptic NMDAr, the mean peak amplitudes of at least five NMDAr-mediated eEPSCs evoked at low frequency were determined every 2 min in the control period before the high frequency stimulation, and subsequently throughout the study. In the studies of presynaptic NMDAr, the average peak amplitude of the 5–6 responses before each episode of 3 Hz stimulation was determined. During the period of 3 Hz stimulation the amplitude of the 5–6 largest events was determined and normalized to the average amplitude of the preceding low frequency events prior to it. Inter-event interval (IEI), amplitude, rise (10–90%) and decay times of AMPAr-mediated sEPSCs and mEPSCs were also determined in some studies of presynaptic NMDAr. Events were detected automatically using a threshold-crossing algorithm. Threshold varied from neurone to neurone but was always maintained at a constant level in any given recording. At least 200 events were sampled during a continuous recording period for each neurone under each condition. Cumulative probability distributions of IEI were compared using the Kolmogorov–Smirnoff (K–S) test. The statistical significance of changes in amplitudes and frequencies was determined with a paired *t* test or one-way ANOVA. All error values indicated in the text and figures refer to S.E.M.

Materials

Salts used in preparation of aCSF were 'Analar' grade and purchased from Merck/BDH (UK). All drugs were applied by bath perfusion. NMDA, MK-801 ((+)-dizocilpine maleate), NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), D-2-AP5 (D-2-amino-5-phosphonovalerate), Ro 25-6981 ((α*R*,β*S*)-α-(4-hydroxy-phenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol hydrochloride) and bicuculline methiodide were obtained from Tocris (UK). TTX came from Alomone Laboratories (Israel).

Results

Control studies

Studies of both pre- and postsynaptic receptor mobility (see below) rely on monitoring recovery after blockade of NMDAr with MK-801. Experiments to determine the rate of washout of the blocker from the slices after a brief period (5 min) of bath perfusion are illustrated in Fig. 1*A*. During perfusion with MK-801 ($n = 4$), isolated NMDAr-mediated eEPSCs were blocked when repetitive stimulation was applied at 2 Hz. A similar stimulation protocol applied 2 min after the start of washout ($n = 3$) was equally effective, showing that sufficient MK-801 was still available in the bath/slices to exert a use-dependent block of the receptors. However, when stimulation was delayed by 7 min after the start of washing ($n = 3$) responses were only reduced by around 50–60%. After 12 min, repetitive stimulation was much less effective ($n = 3$) and with a further 5 min delay (17 min, $n = 3$) blockade was virtually absent. The data

indicate that MK-801 washes relatively rapidly (around 15 min) from the bath/slices, following brief applications at the perfusion rate and bath volume employed in all experiments described below.

We also determined the stability of postsynaptic NMDA-mediated responses in seven neurones (Fig. 1*B*). During repetitive stimulation (MK-801 absent), the slow eEPSCs decreased in amplitude by around 10–30%. Control amplitudes recovered within 3–4 min. Subsequently there was a weak but consistent increase over the next 15–25 min, although this did not reach significance at any point.

Figure 1*C* shows the stability of the frequency-dependent facilitation of AMPAr-mediated eEPSCs used to monitor presynaptic NMDAr-mediated activity ($n = 8$). MK-801 was present in the patch solution in

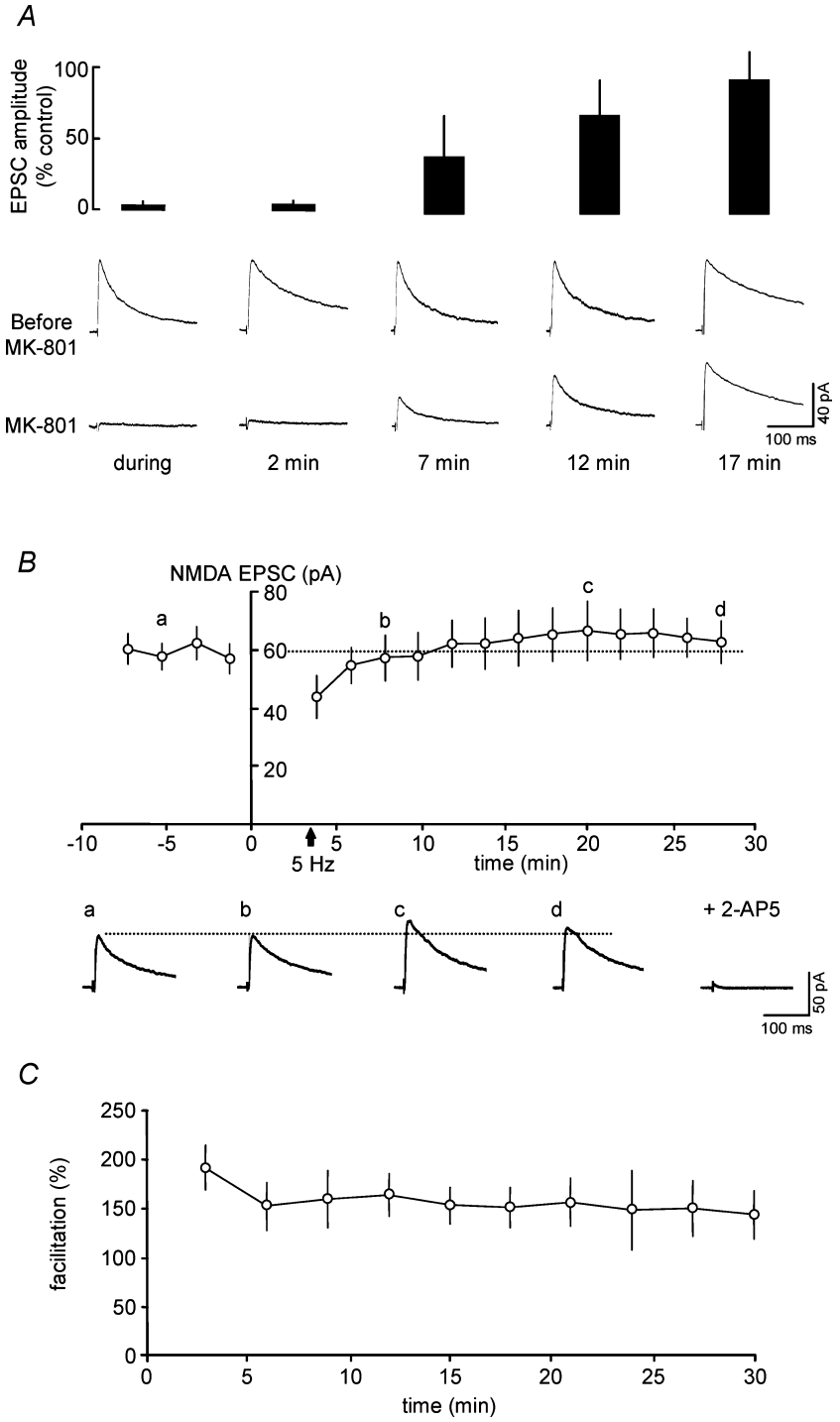

Figure 1. Washout of MK-801 and stability of NMDAr-mediated responses
A, isolated NMDA EPSCs were recorded in the presence of NBQX and bicuculline at a holding potential of +40 mV. Responses were evoked by repetitive stimulation (2 Hz, 40–50 s) before the application of MK-801 (5 min), during its application and at various time points after. The bars show the average reduction in eEPSC amplitude at each time point. The responses below are averages of 6–7 responses recorded in selected individual neurones at each corresponding time point, before or after application of MK-801. The time course of recovery of events at the 7 and 12 min time points is shown in Fig. 2*C*. *B*, stability of postsynaptic NMDAr EPSCs in the absence of MK-801. Each point is the average of 6–8 responses. During repetitive stimulation at 5 Hz for 40–50 s, eEPSC amplitude decreased overall, but recovered to control levels within 3 min. Thereafter, responses were stable. The slight increase noted around 15–25 min was consistent, but not significant. *C*, stability of frequency-dependent facilitation of AMPAr eEPSCs. Frequency-facilitation was quantified as described in the text (low frequency stimulation at 0.05 Hz interleaved with episodes of stimulation at 3 Hz for 5 s) and monitored at 3 min intervals. The graph shows pooled data from 8 neurones. There was an initial decline in facilitation after the first episode but thereafter it remained stable. Stimulation artefacts in the records shown here and in all subsequent figures have been partly blanked for clarity.

four of these studies but absent in the remainder. As there was no detectable difference between groups they were pooled. There was a small but consistent decline in facilitation between the first and second episodes of repetitive stimulation, but thereafter it remained stable throughout the 30 min of recording.

Mobility of postsynaptic NMDAr

The pharmacological approach (see Tovar & Westbrook, 2002; Harris & Pettit, 2007; Zhao *et al.* 2008) used utilizes the properties of blockade by MK-801 to assess mobility of postsynaptic NMDAr. In our first set of experiments we recorded isolated NMDAr-mediated eEPSCs. In these experiments, MK-801 was *not* included in the patch pipette. As noted above, these responses were stable when evoked at low frequency, but rapidly reduced or abolished when the stimulation frequency was increased to 5 Hz in the presence of bath-applied MK-801.

The results from one experiment are shown in Fig. 2*Aa*. The responses were recorded in the presence of bicuculline and NBQX. Each trace is the average of 5–6 responses. Stimulation at 5 Hz in the presence of MK-801 dramatically reduced the NMDAr EPSC. Responses recorded 2 min and 35 min after the start of washout of MK-801 show that they remain blocked and there is no evidence for recovery of the post-synaptic NMDAr-mediated eEPSCs. The time course of experiments in nine neurones is shown in Fig. 2*Ab* and clearly shows the lack of recovery of responses at any point during washout (up to 70 min in some neurones). The use-dependent nature of MK-801 antagonism means that only receptors accessed by glutamate at the activated synapses will be blocked. However, the failure of the eEPSCs to recover during washout suggests that these are not replaced by non-blocked receptors moving into the synapses from distal extrasynaptic sites or by receptors from cytoplasmic storage sites. Note also that all stimulation was halted for a period of 15 min during washout (dotted line), but that responses remained at close to zero when it was restarted. Although control experiments (Fig. 1*A*) clearly show that there is little MK-801 remaining in the bath/slices after 15 min, we wanted to check the possibility that failure to recover was due to residual MK-801, or to rebinding of MK-801 recently dissociated from the receptors. The fact that recovery was absent whether or not stimulation was delivered indicates that this was not the case. Finally, although our control experiments to monitor stability of the NMDAr test responses (Fig. 1*B*) only extended to 30 min compared to the 70 min tested here, it was clear that the lack of recovery could not be attributed to an extensive or permanent rundown of the postsynaptic responses. Thus, the experiments suggest that mobility of NMDAr at these synapses in the EC is limited, or that if it does occur, it may be much slower compared to CA1 (see Tovar & Westbrook, 2002; Zhao *et al.* 2008). However, it should also be noted that Harris & Pettit (2007) have conducted similar experiments and found no evidence for receptor mobility in CA1.

We performed a second set of experiments ($n = 9$) with a slightly different approach and these are illustrated in Fig. 2*B*. In these neurones MK-801 was included in the patch pipette instead of being bath applied. Internal dialysis with MK-801 via the patch pipette begins following whole-cell access, but NMDAr EPSCs could still be evoked over the control period of recording with the stimulation at low frequency (0.05 Hz; Fig. 2*B*), although there was a slow, progressive decline in amplitude. This was rapidly accelerated when high frequency (5 Hz) stimulation was applied, as a result of the use-dependent block of the postsynaptic receptors. On average, the reduction in amplitude of the slow eEPSC by internal MK-801 ($84 \pm 7\%$) was slightly less than that seen with bath-applied MK-801 ($94 \pm 4\%$), suggesting that some postsynaptic NMDAr may have remained unblocked. However, as was the case with bath-applied MK-801, a return to low frequency stimulation was not associated with recovery of the eEPSCs, whether stimulation was applied or not (Fig. 2*B*). In these experiments we confirmed that the small residual eEPSC was NMDAr-mediated by application of 2-AP5. These data support the suggestion that the mobility of postsynaptic NMDAr is low. They also show that this lack of mobility is a characteristic of the synapses on the layer V neurones themselves, since only the NMDAr in the recorded neurones will be blocked and any NMDAr-mediated poly-synaptic events will remain unaltered.

One possible explanation for the lack of mobility could be that the repetitive stimulation is sufficient to cause spillover of glutamate resulting in blockade of both synaptic and extrasynaptic receptors. Harris & Pettit (2008) showed recently that significant recruitment of extrasynaptic NMDAr at synapses in CA1 required stimulation at 25 Hz or above, although they utilized much shorter trains of stimuli. Of course, the situation may be different at layer V synapses, but by limiting the stimulation frequency to 5 Hz in our experiments we hoped to avoid blockade of extrasynaptic receptors and so give ourselves the best chance of detecting receptor mobility. It should be noted that, in the experiments where MK-801 was present internally, there was a small, NMDAr-mediated EPSC remaining after the period of 5 Hz stimulation, suggesting that some of the postsynaptic receptors were not blocked. However, there was still no evidence of recovery during the subsequent 60 min. In addition, in the experiments where we monitored MK-801 washout (Fig. 1*A*) we used a lower stimulation frequency (2 Hz). When stimulation was delayed until

the blocker was already being removed from the bath the residual NMDAr eEPSC clearly indicated that a substantial proportion of the postsynaptic NMDAr (whether synaptic or extrasynaptic) were unblocked, but still there was no recovery (Fig. 2*C*). Finally, Scimemi *et al.* (2004) showed that glutamate spillover could result in MK-801-induced blockade of NMDAr at synapses some distance apart, even when stimulation was delivered at only 0.25 Hz. We have found that if 5 Hz stimulation is replaced with low frequency (0.05 Hz) stimulation at layer V synapses, substantial blockade of postsynaptic NMDAr does not occur within the 5 min perfusion of MK-801 employed

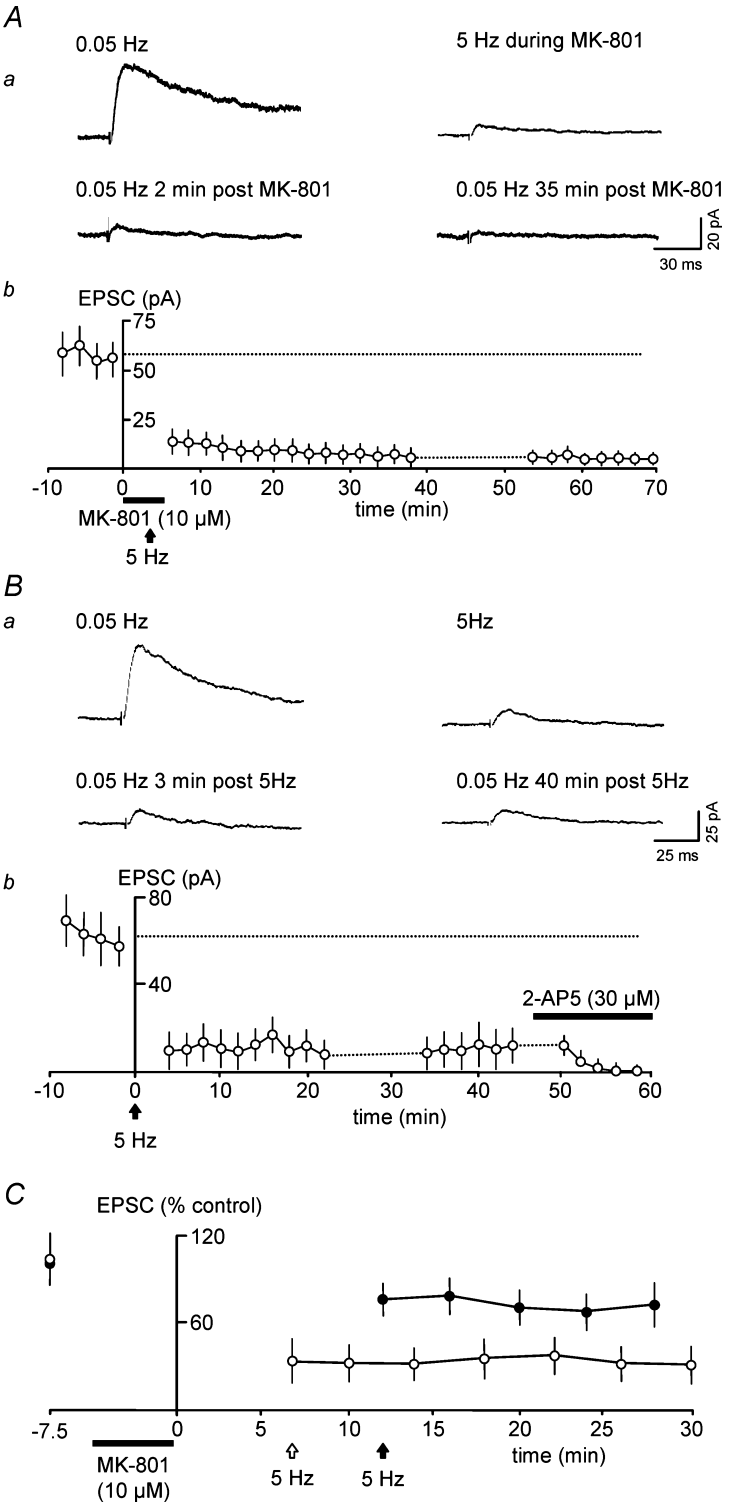

Figure 2. Lack of mobility of postsynaptic NMDAr
Aa, synaptic responses evoked at +40 mV in the presence of NBQX (10 μM) and bicuculline (20 μM), but *without* MK-801 in the patch pipette. The NMDAr-mediated EPSC (0.05 Hz, 5 events averaged) was dramatically reduced by stimulation at 5 Hz for 40–50 s (last 5 events of train averaged) in the presence of bath-applied MK-801. After MK-801 was washed out and stimulation restored to 0.05 Hz there was no recovery of the responses (5 events averaged in each case) in the short or long term. *Ab,* time course of experiments pooled from 9 neurones. Each point is the average of mean amplitudes of EPSCs at each time interval in the 9 neurones. At some of the later time points (60 min plus) not all neurones are included as some recordings were lost at this stage. During the period indicated by the dotted line, low frequency (0.05 Hz) stimulation was halted. *Ba,* similar protocol to *A*, except that MK-801 was included in the patch pipette and omitted from the bath perfusion. The results were essentially the same. *Bb*, pooled data from 9 neurones. In these experiments there was usually a small residual EPSC, which was blocked by 2-AP5. *C,* time course data for the neurones at the 7 min (○, $n = 3$) and 12 min (●, $n = 3$) time points illustrated in Fig. 1*A*. No recovery of eEPSC amplitude was seen during the washout of MK-801.

in our studies, and requires perfusion periods of around 20 min, with the attendant problems of a much extended period of washout. However, we have done this in two neurones and found that even with the very low frequency there is no suggestion of any recovery over a subsequent 35 min of washout of MK-801 (not shown). Thus, we feel confident that the lack of recovery of the NMDAr response is due to a lack of mobility rather than due to a global blockade of all postsynaptic receptors.

Mobility of presynaptic receptors

Unless otherwise indicated, MK-801 was included in the patch pipette to block postsynaptic NMDAr in these experiments. Under these conditions eEPSCs are mediated primarily by postsynaptic AMPAr (Berretta & Jones, 1996; Woodhall *et al.* 2001). As noted above, we used frequency-dependent facilitation of AMPAr-mediated eEPSCs (Woodhall *et al.* 2001; Chamberlain *et al.* 2008) as a reporter of the function of presynaptic NMDAr in order to investigate their mobility. To quantify frequency-facilitation, responses evoked at low frequency (0.05 Hz) were interspersed with periods of high frequency stimulation, and response amplitudes during the latter normalized to the average amplitude of the preceding low frequency events. When frequency-dependent facilitation was stable, it was then blocked by bath perfusion of MK-801 for 5 min, and the blocker then washed out. Alternate periods of low and high frequency stimulation were continued uninterrupted throughout the application of MK-801 and washout period.

In the first set of experiments we examined changes in facilitation, recorded uninterrupted, after a brief application of MK-801 in 10 neurones. Averaged eEPSCs from one neurone are illustrated in Fig. 3*A*. The frequency-dependent facilitation of eEPSCs during 3 Hz stimulation is readily apparent. During application of MK-801 this was abolished but low frequency responses were unaffected. However, in contrast to the postsynaptic NMDAr-mediated responses, it is clear that after MK-801 was washed out, frequency-facilitation mediated by presynaptic NMDAr progressively recovered towards control levels over a period of 30–40 min. The time course of this recovery in 10 neurones is shown in Fig. 3*B*. Initially, the average change in response amplitude during 3 Hz stimulation became slightly negative because a weak frequency-dependent depression of eEPSCs was often seen when facilitation was blocked. However, this was quickly replaced by facilitation again, which progressively increased after MK-801 was washed out. Facilitation was more variable than prior to application of the blocker, but had returned to control levels by 40–45 min. Note that these experiments, and those below employed the same recording chamber, perfusion rate and period of application of MK-801 employed in the experiments on postsynaptic NMDAr EPSCs. Although we have not conducted experiments using frequency-facilitation to monitor MK-801 washout we would expect that the blocker would have a similar time course of availability at both the presynaptic receptors and postsynaptic receptors and that washout of MK-801 would be complete after about 15 min. Frequency-facilitation was monitored in the absence of any drugs (Fig. 1*C*) and was stable over at least 30 min, so state-dependent alterations in presynaptic receptor sensitivity were unlikely to influence recovery.

Thus, anomalous recovery of frequency-dependent facilitation provides a strong indication that NMDAr in the presynaptic membrane may be relatively mobile, particularly compared to their postsynaptic counterparts. Presynaptic NMDAr, close to the release sites and activated by glutamate, will be use-dependently blocked by MK-801 during repetitive stimulation. They should remain blocked during and after washout of MK-801, as its binding in the channel is essentially irreversible. The anomalous recovery of facilitation suggests that the blocked presynaptic receptors may be replaced at the release sites by others that were originally outside the range of the released glutamate, did not undergo a use-dependent block, and were able to move into the vicinity of the release sites.

An alternative possibility is that the presynaptic NMDAr are stably anchored at release sites, and that recovery of facilitation occurred as a result of use-dependent unblocking of these receptors by glutamate released as a result of stimulation in the washout period. If this were the case we would expect that halting stimulation would negate recovery during this period, and that recovery would only occur when the stimulation was recommenced. We examined this possibility in seven neurones. Results from one neurone are illustrated in Fig. 4*A*. Facilitation was again abolished by bath application of MK-801. Shortly following the start of the washout period, all stimulation (both high and low frequency) was halted for 15 min. However, when stimulation was recommenced, it was clear that recovery had occurred despite the hiatus. Summary data for the seven neurones is shown in Fig. 4*B*. Allowing for the period of no stimulation, the time course of recovery was remarkably similar to that seen in neurones where the stimulus protocol was unchecked throughout (Fig. 3). In these experiments we applied NMDAr antagonists after recovery to confirm that the facilitation seen during anomalous recovery was due to activation of presynaptic NMDAr. In four neurones we used 2-AP5 (30 μM) and this rapidly abolished facilitation. We have recently shown that frequency-dependent facilitation in layer V is mediated by NMDAr containing the NR2B subunit (Chamberlain *et al.* 2008). In three further studies we used the NR2B-selective antagonist, Ro 25-6981 (500 nM), and this also abolished facilitation (see Fig. 7*A*), showing that the presynaptic receptors that replace those

following MK-801 blockade are likely to be the same as those initially present at release sites. Because 2-AP5 and Ro 25-6981 had the same effect these studies have been pooled in Fig. 4*B*.

The use-dependent nature of MK-801 blockade means that only receptors accessed by glutamate in the vicinity of the release sites will be blocked. Recovery would then result from non-blocked receptors moving from distal inaccessible sites on or in the terminals. It follows then that if we are able to globally block these receptors then recovery should not occur. We investigated this in a third set of experiments. We combined bath application of MK-801 with concurrent bath application of NMDA ($25\ \mu$M) to globally block surface-expressed NMDAr. Results from one neurone, together with pooled data from the seven neurones tested, are shown in Fig. 5. The concurrent application of MK-801 and NMDA rapidly abolished frequency-dependent facilitation. However, prolonged washout of both agents was not accompanied by anomalous recovery. Thus, these studies provide strong evidence that the recovery of facilitation shown in the previous experiments was due to the presence of non-blocked receptors in the distal terminal membrane, able to move into the proximity of the glutamate release

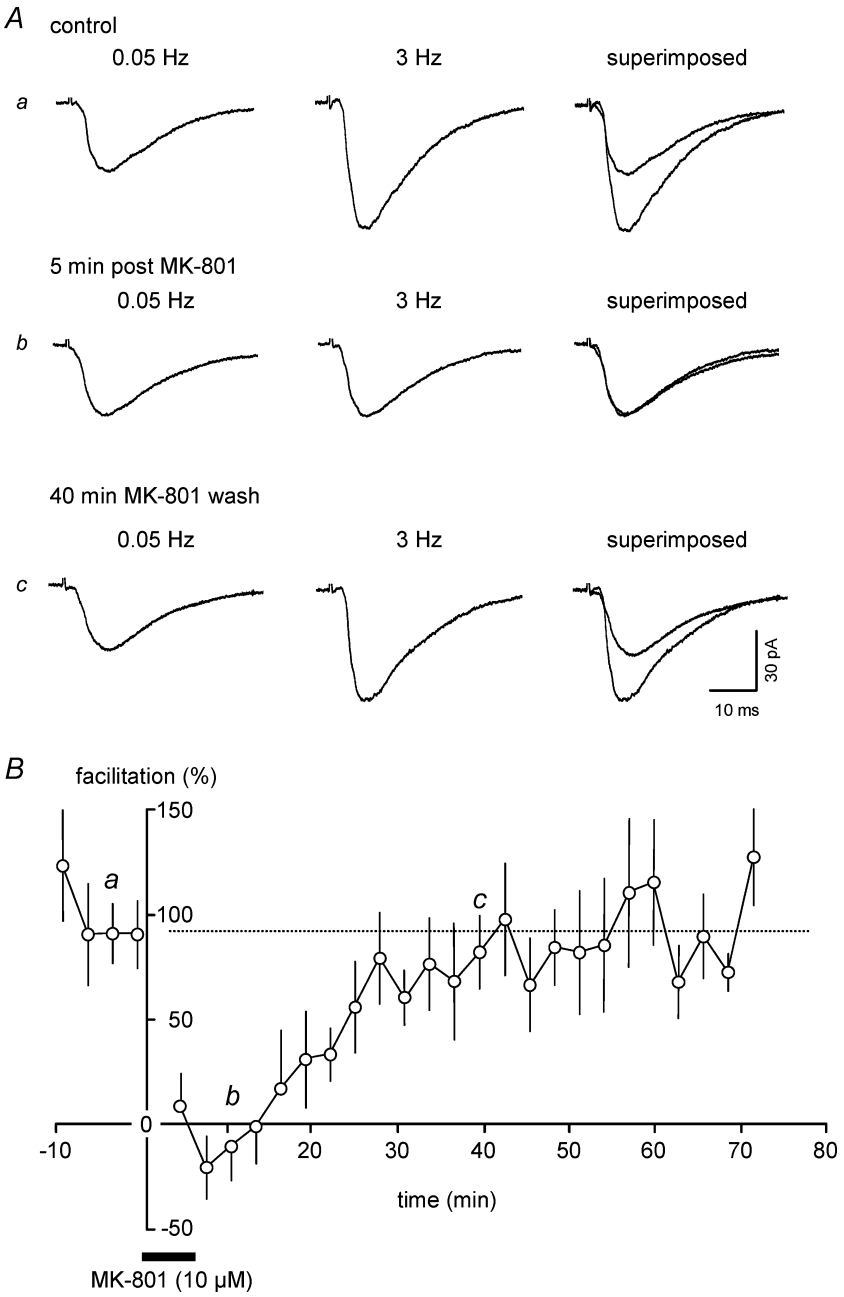

Figure 3. Mobility of presynaptic NMDAr
The responses are AMPAr-mediated eEPSCs (with MK-801 in patch pipette, average of 5 responses) evoked with interleaved low (0.05 Hz) and high (3 Hz, 5 s every 2 min) frequency stimulation. *A*, there was a clear frequency-dependent facilitation of the AMPAr eEPSCs during 3 Hz stimulation (*a*). During, and shortly following, bath application of MK-801 (*b*), the amplitude of low frequency responses was unaffected, but the frequency-facilitation was largely abolished. However, responses recorded after 40 min (*c*) show a full recovery of facilitation. *B,* time course of studies pooled from 10 neurones. Blockade of frequency-facilitation by MK-801 revealed a brief, weak overall frequency depression of eEPSCs followed by a progressive anomalous recovery.

sites. Receptors located in cytoplasmic storage sites would not be accessible to either agonist or blocker so they would remain unblocked during perfusion with the two drugs. Thus, the fact that no recovery was seen after the combined treatment strongly suggests that the recovery seen with MK-801 alone reflects lateral diffusion of NMDAr already inserted in the terminal membrane.

We cannot completely rule out the possibility that anomalous recovery of facilitation reflects MK-801 unbinding from a proportion of presynaptic NMDAr. However, the experiments above suggest this is very unlikely. Tovar & Westbrook (2002) investigated this possibility by transiently applying the competitive antagonist 2-AP5 during recovery from MK-801 to prevent use-dependent dissociation of the channel blocker. This manipulation did not alter the rate of recovery. We considered a similar approach in our studies of presynaptic NMDAr, but ruled it out because of technical difficulties associated with our experimental situation. Tovar & Westbrook (2002) used a highly reduced culture preparation and a fast perfusion system for drug application capable of solution exchanges in the vicinity of the recorded cell in 30–40 ms, allowing perfusion of the competitive antagonist to be turned on and off almost instantaneously. That is not possible in native tissue with an extracellular perfusion system. Recovery of responses from even a short period of perfusion with

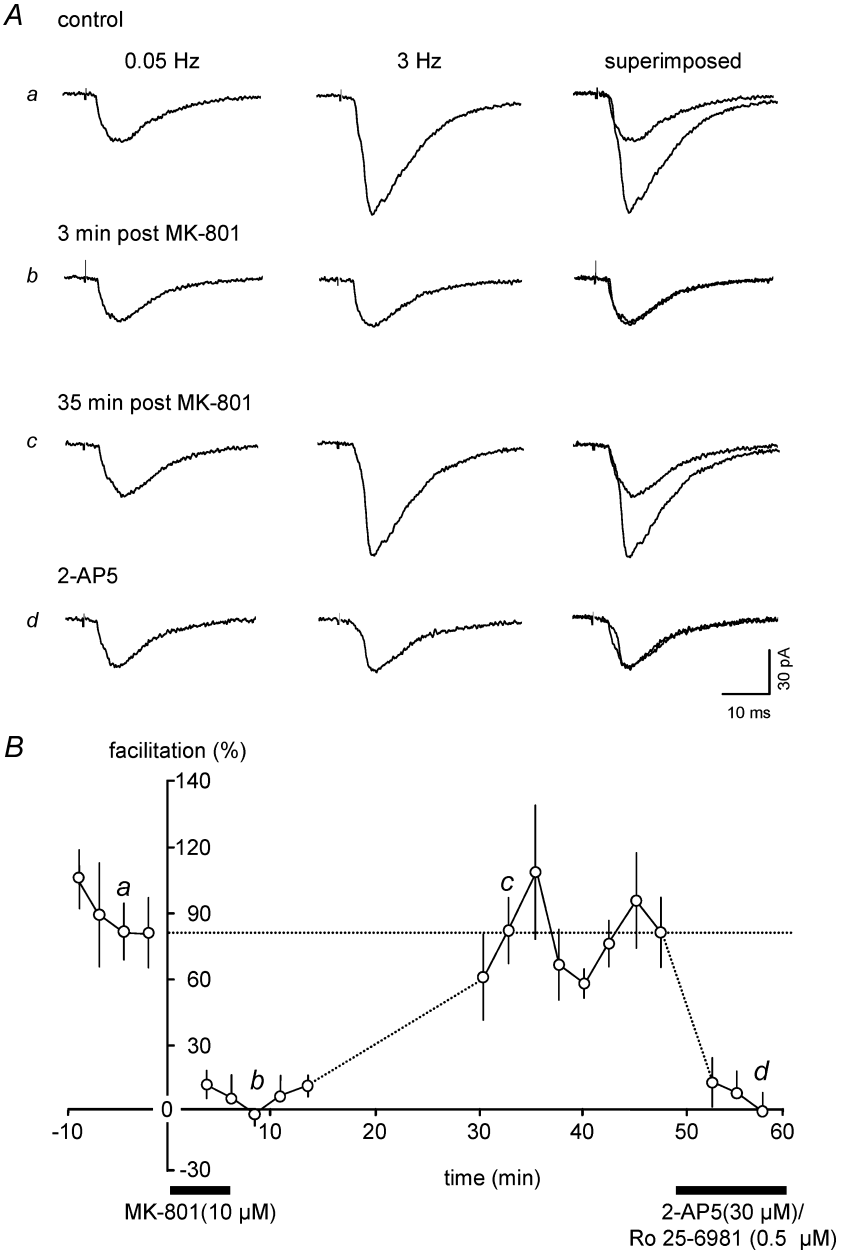

Figure 4. Anomalous recovery is not dependent on continuous stimulation
The protocol was the same as in Fig. 2, where low frequency-stimulation (0.05 Hz) was interleaved with 3 Hz stimulation (3 Hz, 5 s, every 2 min). *A,* frequency-facilitation of AMPAr-mediated eEPSCs (*a*) is again blocked in the presence of MK-801. Full recovery was seen after 35 min (*c*) despite the fact that no stimulation was delivered between 15 and 30 min. *B*, time course of the studies pooled from 7 neurones. In these neurones, after anomalous recovery, we applied either 2-AP5 ($n = 4$) or Ro 25-6981 ($n = 3$), either of which blocked the recovered frequency-dependent facilitation.

2-AP5 is gradual (see Fig. 7*B*), and in our experimental set-up, recovery from competitive blockade would simply be superimposed on the recovery due to (presumed) receptor mobility, and would not confirm or deny the possibility that unbinding of MK-801 was occurring. However, we believe that the lack of anomalous recovery following global blockade of facilitation with combined application of MK-801 and NMDA, and the failure of halting stimulation to alter the rate of recovery, strongly suggest that use-dependent unbinding of the blocker is unlikely to be responsible for recovery. This conclusion is also indirectly supported by the lack of recovery of postsynaptic NMDAr function after blockade with MK-801.

Activity-dependent changes in presynaptic receptor function

We have shown previously that presynaptic NMDAr in the EC are tonically activated by ambient glutamate, since antagonists induce a decrease in spontaneous glutamate release (Berretta & Jones, 1996; Woodhall *et al.* 2001; Yang *et al.* 2006, 2007). Therefore, we predicted that changes in frequency-facilitation of eEPSCs might be paralleled by changes in spontaneous glutamate release. Thus, we determined changes in glutamate-mediated sEPSCs in the neurones illustrated in Figs 3 and 5.

In the neurones where MK-801 alone was bath applied, blockade of frequency-dependent facilitation (Fig. 3) was

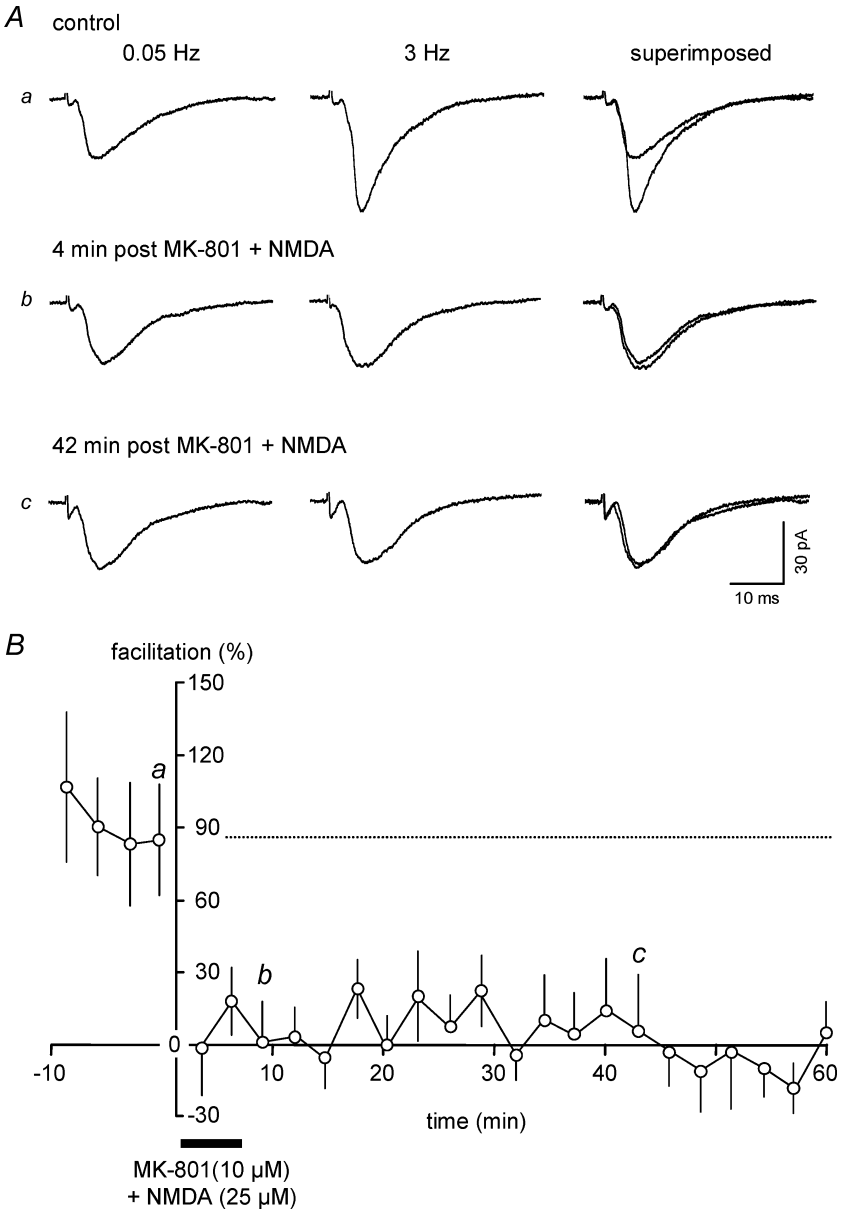

Figure 5. Anomalous recovery does not occur after global blockade of NMDAr
The protocol was the same as in Fig. 2, where low frequency stimulation (0.05 Hz) was interleaved with 3 Hz stimulation (5 s, every 2 min). *A*, the records in one neurone show that frequency-facilitation is abolished by combined application of MK-801 together with NMDA, again with no detectable effect on low frequency responses (*b*). However, now, despite prolonged washing of both agents, there was no evidence for anomalous recovery of facilitation (*c*). *B*, time course of experiments pooled from 7 neurones.

accompanied by a substantial decrease in frequency of sEPSCs (Fig. 6*Aa*). Mean IEI increased from a control value of 306 ± 80 ms to 615 ± 136 ms at the start of the washout period. When facilitation of eEPSCs had returned to control levels 40 min later, mean IEI decreased to 264 ± 75 ms, These changes reflect a decrease in frequency of around 50% by MK-801 and an increase of about 15% associated with anomalous recovery. K–S analysis of cumulative probability distributions of IEI showed that both the increase after MK-801 ($P < 0.001$) and decrease

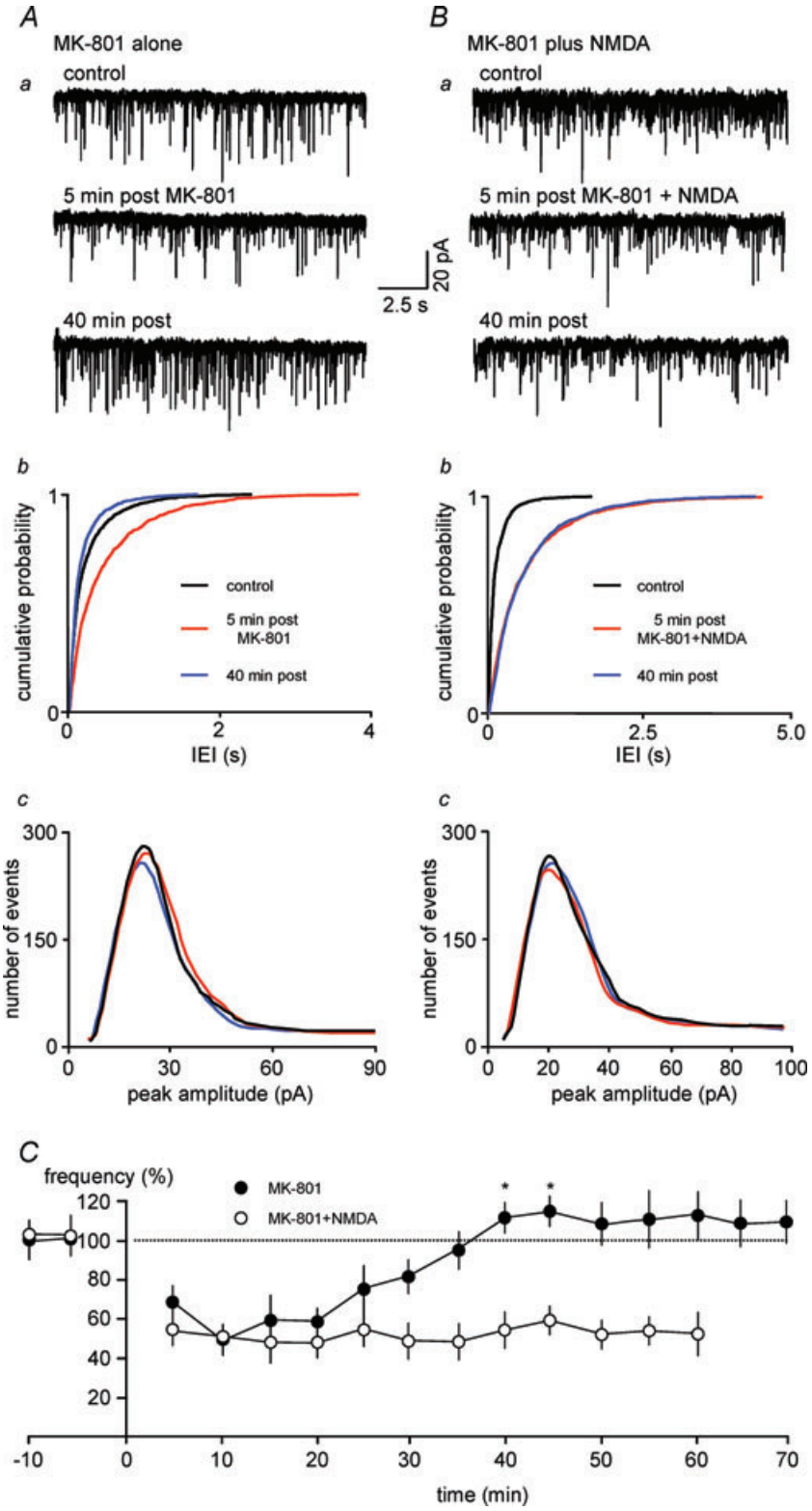

Figure 6. Anomalous recovery is paralleled by changes in sEPSCs
A, sEPSCs were recorded in the 10 neurones included in Fig. 3. The recordings from one neurone shown in *Aa* were obtained before application of MK-801, 5 min after washing the drug from the bath, and 40 min after washout, when recovery of frequency-dependent facilitation was complete. Cumulative probability analysis of pooled data of IEI from all 10 neurones is shown in *Ab* and the distribution of peak amplitudes of events at the same time points is shown in *Ac*. Blockade of frequency-dependent facilitation of eEPSCs by MK-801 (Fig. 3) was accompanied by a decrease in frequency of sEPSCs (rightward shift in IEI cumulative probability distribution) with little overall change in amplitude. Recovery of frequency-facilitation of eEPSCs (see Fig. 3) was accompanied by an increased sEPSC frequency (leftward shift in IEI) with again little overall change in amplitude although there was a slight shift towards larger amplitudes during recovery. *B,* analysis of sEPSCs in the 7 neurones illustrated in Fig. 5. Combined application of NMDA and MK-801 shifted the cumulative probability distribution of sEPSC IEI to the right, reflecting a decrease in frequency of events. However, after 40 min of washout of both agents, there was no recovery of sEPSC frequency. Again there was little change in amplitude distributions. *C*, time course of changes in sEPSCs in the two groups. For this analysis, mean IEI of all events in 5 min blocks was determined and converted to frequency for each neurone. Changes in frequency were determined as a percentage of the mean control frequency at each time point, and averaged across all neurones in each group.

after washout ($P < 0.01$) were significant compared to control (Fig. 6*Ab*). There was no significant change in mean amplitudes (control: 15.5 ± 3.1 pA; MK-801: 14.0 ± 3.1 pA; wash: 14.0 ± 2.9 pA), and little overall change in frequency distribution of peak amplitudes (Fig. 6*Ac*). Mean rise times (1.5 ± 0.1 *versus* 1.6 ± 0.1 *versus* 1.6 ± 0.3 pA) and decay times (control: 3.5 ± 0.5 *versus* 3.7 ± 0.3 *versus* 3.7 ± 0.2 ms) were not significantly altered at any stage, suggesting that the changes in sEPSCs occurred as a consequence of the blockade and recovery of presynaptic NMDA receptors.

The effects on sEPSCs differed when MK-801 was bath applied in conjunction with NMDA (same 7 neurones as illustrated in Fig. 5). In this case, sEPSC frequency was reduced but failed to recover during washout. In the control situation, IEI was 251 ± 41 ms. Five minutes following the washout of the agonist and the blocker, IEI was increased to 552 ± 76 ms, again reflecting a decrease in frequency of around 50%. After 40 min of washing, IEI remained elevated at 598 ± 91 ms (Fig. 6*Aa*,*b*). Thus, the failure of frequency-dependent facilitation of eEPSCs to recover following global blockade of NMDAr was paralleled by a reduction in tonic facilitation of spontaneous release, which likewise failed to recover. Again, mean amplitudes were unaffected throughout (control: 12.8 ± 1.8 pA; MK-801: 12.5 ± 1.5 pA; wash: 13.3 ± 1.7 pA) and there was little change in amplitude distributions (Fig. 6*Bc*). Mean rise (1.6 ± 0.2 *versus* 1.9 ± 0.2 *versus* 1.7 ± 0.1 pA), and decay times (control: 4.2 ± 0.5 *versus* 5.3 ± 0.6 *versus* 5.1 ± 0.5 ms) were again unaffected.

Figure 6*C* shows the time course of changes in sEPSC frequency in the two sets of experiments. It is clear that recovery was not seen after global blockade of presynaptic NMDAr, but did occur when only NMDAr accessible to synaptically released glutamate were blocked. The increase above control levels was small, but consistent and persistent during the period of recording, although it only reached significance at two time points. This increase in spontaneous glutamate release during anomalous recovery of frequency-dependent facilitation (Fig. 6*Aa*,*b*) could be indicative of an activity-dependent increase in trafficking of NMDAr when those receptors close to the release sites are compromised. Facilitation of eEPSCs did become much more variable after recovery from MK-801. Although there was no significant overall increase in frequency-dependent facilitation of eEPSCs there was a relatively clear increase in at least five neurones, three in experiments when stimulation was uninterrupted (Fig. 3) and two when it was halted during recovery (Fig. 4). An example of the latter is shown in Fig. 7*A*. In this neurone, frequency-facilitation was around 50% in control, and this progressed to around 80%, 40 min after washout of MK-801. In this neurone facilitation was abolished by Ro 25-6891, demonstrating that it was likely to be mediated exclusively by NR2B-containing receptors.

To look further at this enhancement of presynaptic NMDAr activity after receptor blockade, we determined the effect of a short period of competitive NMDAr blockade in a group of 6 neurones using a similar protocol to that used to monitor recovery from MK-801 blockade. In these experiments MK-801 was included in the patch pipette but 2-AP5 was substituted for bath application of MK-801 to abolish frequency-dependent facilitation ($n = 5$). The results of these studies are summarized in Fig. 7*B*. As expected, 2-AP5 abolished frequency-facilitation and this recovered relatively rapidly over 15–20 min when the drug was washed out. However, there was also an increase in frequency-facilitation beyond control levels following recovery, which then declined back towards control over a further 20–30 min. The increase in facilitation was significant ($P < 0.05$) at several points. As noted, frequency-facilitation is mediated primarily by NR2B receptors (Woodhall *et al.* 2001; Chamberlain *et al.* 2008), and in preliminary experiments ($n = 2$, not shown) we have shown that brief blockade with Ro 25-6891 is also followed by a weak increase above control during recovery. Thus, the data do suggest that an overall reduction of activation of presynaptic NMDAr, presumably NR2B, could initiate an accelerated diffusion of receptors towards the release sites.

Finally, we have determined whether the opposite may be true by applying NMDA alone in the absence of external MK-801. Application of NMDA (25 μM) often resulted in the generation of large recurrent bursts of oscillatory activity, which appeared to be network driven, making meaningful analysis of sEPSCs and eEPSCs problematic. In addition, eEPSCs *per se* were increased in amplitude as might be expected, but more importantly, the agonist resulted in a change in profile of activity at 3 Hz from facilitating to depressing, or a variable mix of facilitation and depression. To avoid these complications, in the current experiments we examined mEPSCs recorded in the presence of TTX (1 μM). A brief application of NMDA ($n = 4$) resulted in a substantial decrease in IEI of mEPSCs from 213 ± 44 ms to 125 ± 27 ms, reflecting an increase in frequency from around 4.5 Hz to 8 Hz. However, after washout of the agonist, mEPSC frequency then declined to below baseline levels where it remained for at least 45 min. IEI at 25 min was 320 ± 66 ms and at 45 min it was 330 ± 66 ms reflecting a frequency of approximately 3.0 Hz at both time points.

The results of these studies are summarized in Fig. 8. Figure 8*B* shows analysis of IEI and peak amplitudes of sEPSCs in the neurone illustrated in Fig. 8*A*. Both the leftward shift in cumulative probability distribution elicited by NMDA, and the subsequent rightward shift during recovery, were highly significant (K–S test, $P < 0.001$). The frequency distribution of event

amplitudes was not greatly altered by NMDA, or during recovery from it. Mean control amplitude in this neurone was 23.3 ± 0.5 pA, 21.9 ± 0.4 pA during NMDA, 22.3 ± 0.4 after 25 min recovery and 23.5 ± 0.5 after 45 min. Pooled data from the four neurones tested are shown in Fig. 8*C*, and these largely reflect those seen in the neurone in Fig. 8*A*. However, overall there was a small shift towards slightly larger amplitude events in the presence of NMDA, reflected by a small, but significant ($P < 0.05$) increase in mean peak amplitude from 16.8 ± 0.4 pA in

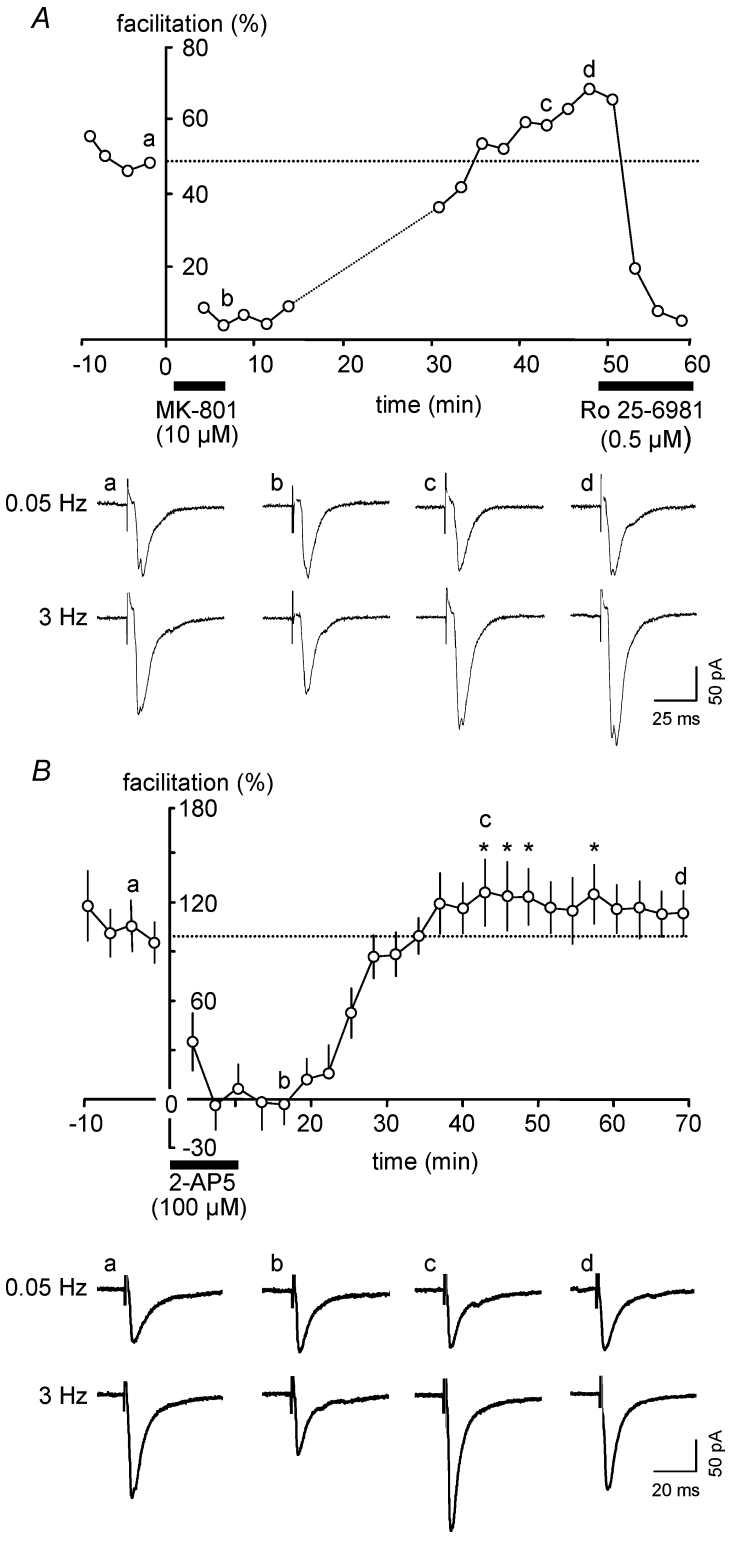

Figure 7. Blockade of NMDAr may promote presynaptic NMDAr mobility
A, results from one neurone included in the analysis of Fig. 4. Stimulation at 3 Hz was delivered for 5 s every 2 min. Again, each point represents the mean facilitation occurring during these periods relative to the mean amplitude of responses at 0.05 Hz in the intervening periods. Following anomalous recovery, frequency-dependent facilitation increased beyond control levels. The NR2B-selective antagonist Ro 25-6981 abolished the enhanced facilitation. *B*, in 5 neurones 2-AP5 was used to a block frequency-dependent facilitation instead of MK-801. Recovery was succeeded by a period where facilitation exceeded that seen in control conditions followed by a gradual decline towards baseline levels by 60–70 min.

control to 18.5 ± 0.5 pA when NMDA was added. Peak amplitudes declined again during recovery to 15.6 ± 0.3 at 25 min and 15.9 ± 0.5 pA at 45 min, slightly, but not significantly, below baseline. The time course of the changes in sEPSC frequency are shown in the graph in Fig. 8*D*, which clearly illustrates the rise induced by NMDA followed by the persistent decrease. Changes in frequency were significant ($P < 0.01$) at all time points.

Discussion

Glutamate release at cortical synapses is facilitated by presynaptic NMDAr (Berretta & Jones, 1996; Woodhall *et al.* 2001; Sjöström *et al.* 2003; Jourdain *et al.* 2007; Li & Han, 2007; Brasier & Feldman, 2008; Li *et al.* 2008). We have now demonstrated that presynaptic NMDAr are likely to be dynamically mobile, and able to exchange between locations close to release sites and more distal sites in the

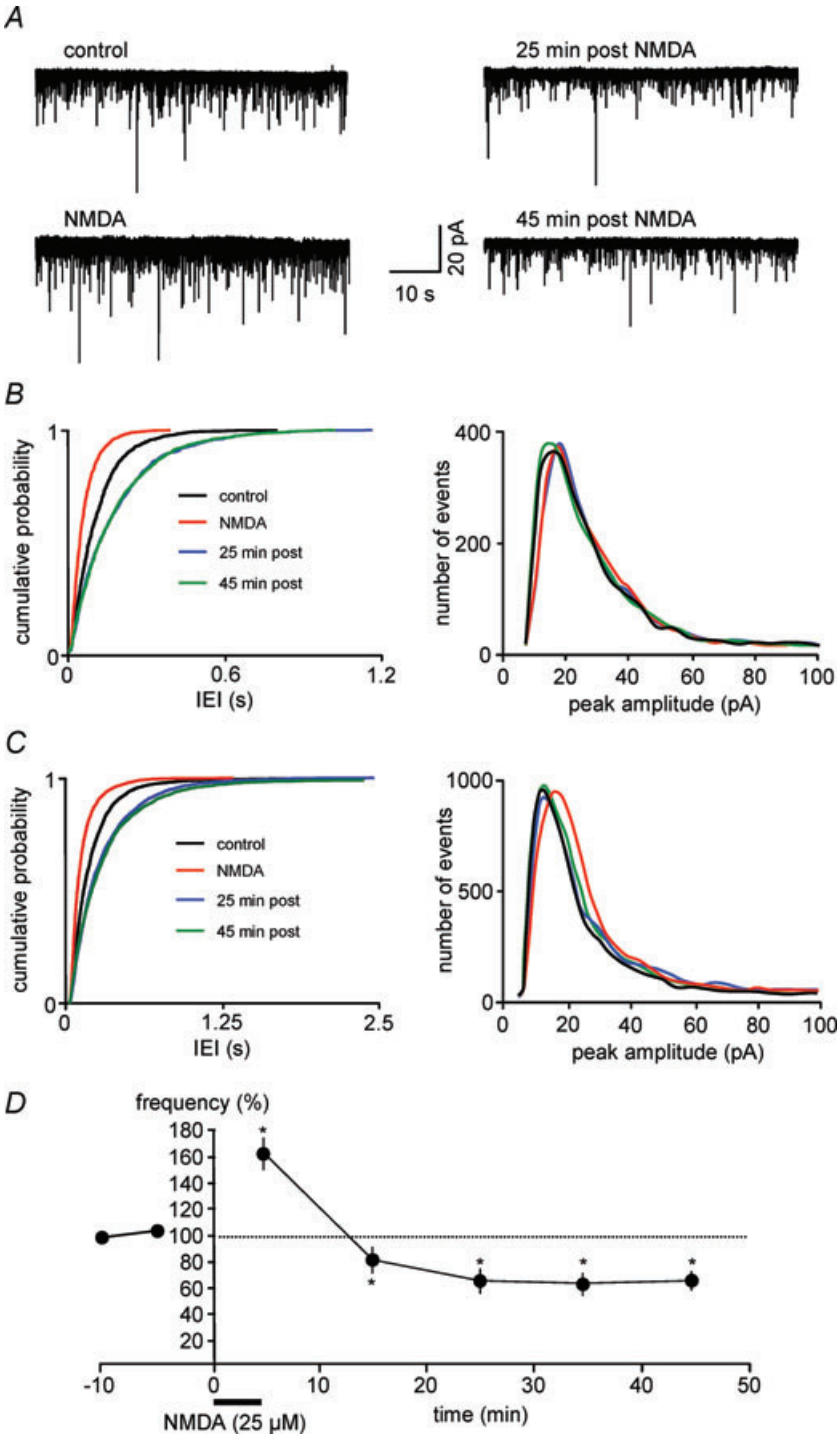

Figure 8. Activation of NMDAr may decrease presynaptic receptor mobility
A, recordings from a layer V neurone showing the effects of a brief (5 min) application of a low concentration (25 μM) of NMDA on mEPSCs recorded in the presence of TTX (1 μM). The agonist caused a substantial increase in mEPSC frequency, but following its washout there was a sustained decrease in frequency over the subsequent recording period (up to 50 min). This is reflected by the analysis of cumulative probability of IEI shown in *B*. Each plot consists of 1500 mEPSCs. The shift to the left in NMDA reflects the change to shorter intervals and a higher frequency. During recovery, the distributions are persistently located to the right of control reflecting the decreased frequency. Frequency histograms of amplitudes of the same events show little change in amplitude distribution at any time point. *C*, IEI and amplitude analysis of data pooled from 4 neurones. Each neurone contributed 500 events to the pooled analysis. The results mirror those in the neurone illustrated in *A* and *B* except that there was a slight shift in amplitude distribution towards larger events in the presence of NMDA. *D*, time course of the effects of NMDA. Mean IEI of all events in 5 min blocks was determined and converted to frequency for each neurone. Changes in frequency were determined as a percentage of the mean control frequency at each time point, and averaged across all neurones.

terminal membrane. In contrast, we found that NMDAr in the postsynaptic membrane are likely to reside in relatively stable synaptic and extrasynaptic pools.

Mobility of postsynaptic NMDAr

There has been increasing interest in the movement of receptors between synaptic and extrasynaptic compartments. Most of this attention has focused on the mobility of postsynaptic receptors as a basis for long-term synaptic plasticity (see Carroll & Zukin, 2002; Collingridge *et al.* 2004; Perez-Otano & Ehlers, 2005; Lau & Zukin, 2007). Trafficking appears to occur in at least two compartments. Receptors may cycle between the synaptic membrane and intracellular sites (see Carroll *et al.* 1999; Collingridge *et al.* 2004; Nong *et al.* 2004; Groc & Choquet, 2006), or by lateral diffusion in the cell membrane without internalization (Choquet & Triller, 2003; Groc *et al.* 2004; Triller & Choquet, 2005; Groc & Choquet, 2006). Both forms of trafficking contribute to functional mobility of NMDAr, and it seems likely that the two probably interact, with receptors moving into and out of the PSD by lateral diffusion, and then recycled via endocytosis at more distal sites in spines (see Washbourne *et al.* 2004; Groc & Choquet, 2006; Lau & Zukin, 2007). NMDAr were originally thought to be stably anchored at the PSD, and that AMPAr receptors were more mobile, However, evidence now suggests that both receptors can be exchanged between the synaptic membrane and internal stores as well as between synaptic and extrasynaptic sites (Collingridge *et al.* 2004; Groc *et al.* 2004; Nong *et al.* 2004; Triller & Choquet, 2005; Groc & Choquet, 2006).

A number of studies have used biochemical, immuno-fluorescence techniques, and direct imaging to monitor postsynaptic receptor trafficking (e.g. Groc *et al.* 2004; Washbourne *et al.* 2004). We have examined functional mobility using the pharmacological approach of monitoring anomalous recovery of NMDAr EPSCs after blockade with MK-801 (Tovar & Westbrook, 2002; Harris & Pettit, 2007; Zhao *et al.* 2008). Tovar & Westbrook (2002) found that NMDAr could move by lateral diffusion between synaptic and extrasynaptic compartments at autaptic synapses in hippocampal cultures, and Zhao *et al.* (2008) reported similar findings in CA1 synapses in hippocampal slices. In contrast, Harris & Pettit (2007) found no exchange of synaptic and extrasynaptic receptors in CA1 pyramidal neurones using the same approach. Clearly our results are in agreement with the latter study, as we found no indication of mobility of postsynaptic NMDAr in the EC, where they appear to be stably anchored or only slowly mobile. Of course, we are studying different synapses to those in previous studies and there is no reason *a priori* to assume that mobility of postsynaptic receptors is a common feature of all glutamate synapses. However, this does not explain the differences between studies in the hippocampus. One possible factor could be a developmental decline in receptor mobility. There is considerable evidence to suggest that subunit composition of postsynaptic NMDAr changes during development, particularly with respect to synaptic *versus* extrasynaptic location and synaptic plasticity (e.g. Wenzel *et al.* 1997; Rumbaugh & Vicini, 1999; Tovar & Westbrook, 1999; Liu *et al.* 2004; Groc *et al.* 2006*b*). Mobility at cultured autaptic synapses (Tovar & Westbrook, 2002) was studied during early development (6 days *in vitro*). Rapid exchange of synaptic NMDAr from NR2B- to NR2A-containing is pronounced at postnatal days (P) 2–9 but declines markedly by P16–21 (Bellone & Nicoll, 2007). Our studies were conducted at P21–35, so it is possible that receptor mobility at EC synapses has already stabilized at this stage. We have recently shown that postsynaptic NMDAr at these synapses may contain a high proportion of triheteromeric NR1/NR2A/NR2B receptors (Chamberlain *et al.* 2008), a situation suggested to represent a stable mature situation (Tovar & Westbrook, 1999). Again, however, this does not explain the contrasting results in hippocampus reported by Harris & Pettit (2007; P14–21) and Zhao *et al.* (2008; P21) in hippocampal slices.

Mobility of presynaptic NMDAr

In contrast to the lack of mobility of NMDAr at postsynaptic sites, using a similar physiological approach we found clear evidence that NMDAr in presynaptic terminals in the EC are highly mobile. The data strongly suggest that this mobility results from lateral diffusion of receptors in the terminal membrane. We cannot entirely dismiss the possibility of trafficking of NMDAr to and from internal stores in the terminals. One scenario is that receptors are translocated from cytoplasmic sites to the terminal membrane at distal locations and subsequently move into the proximity of the release sites and access to synaptically released glutamate by lateral diffusion, analogous to the situation that may occur in postsynaptic spines (Groc & Choquet, 2006; Lau & Zukin, 2007).

Our results provide a first functional demonstration of trafficking of NMDAr in presynaptic terminals. Immuno-labelling studies of NMDAr subunits have shown them located in proximity to the terminal membrane of active zones (Wang & Pickel, 2000; Fujisawa & Aoki, 2003; Adams *et al.* 2004; Kotak *et al.* 2005). However, receptors have also been seen close to the membrane at extra-junctional sites in terminals and axons, as well in the cytoplasm distal to the active zones (e.g. Aoki *et al.* 1994; Conti *et al.* 1999; Wang & Pickel, 2000; Adams *et al.* 2004; Kotak *et al.* 2005; Jourdain *et al.* 2007). This provides physical evidence for the existence of extra-junctional presynaptic NMDAr as a source of receptors to be trafficked to the active zones. Interestingly, immuno-labelling of NR2B subunits has been detected

in association with vesicular organelles in hippocampal terminals (Saldanha *et al.* 2004; Jourdain *et al.* 2007), perhaps representing a storage/delivery mechanism for trafficking of NMDAr to the terminal membrane.

Few previous studies have considered trafficking of presynaptic NMDAr. Electron microscopy combined with immuno-labelling indicated that the number of NR2A and NR2B subunits in terminals in rat somatosensory cortex *in vivo* appeared to increase and decrease, respectively, during NMDAr-blockade (Aoki *et al.* 2003; Fujisawa & Aoki, 2003). This was taken as evidence that presynaptic NR2A and NR2B subunits undergo regulated trafficking in these terminals. Presynaptic NMDAr may originate in the somatic endoplasmic reticulum and be transported along axons to terminals, analogous to the transport of NMDAr to spines along dendrites (see Lau & Zukin, 2007). The anterograde transport of NMDAr along vagal axons was suggested to reflect trafficking of NMDAr into terminals to act as autoreceptors (Cincotta *et al.* 1989). O'Donnell *et al.* 2004) observed NMDAr receptors in the axoplasm of spinal axons and suggested these were being transported along microtubules to primary afferent terminals, where they would also act as autoreceptors (albeit inhibitory; Bardoni *et al.* 2004). Scaffolding proteins such as PSD-95, and SAP-102 (Kornau *et al.* 1995; Niethammer *et al.* 1996; Fujita & Kurachi, 2000) anchor postsynaptic NMDAr at the PSD, and such proteins also occur in cortical terminals (Valtschanoff *et al.* 1999; Aoki *et al.* 2001). Our studies demonstrate a rather dynamic mobility of presynaptic NMDAr, but the presence of scaffolding proteins presynaptically could suggest that receptors that diffuse from distal sites may be anchored near active zones by mechanisms similar to those at the PSD.

We have not yet fully examined the functional role of presynaptic NMDAr mobility. One possibility is a role in activity-dependent plasticity. We found that anomalous recovery from MK-801, or acute blockade with 2-AP5 could be followed by a period of enhanced frequency-dependent facilitation of AMPAr-mediated transmission and of spontaneous glutamate release. These effects could be explained by an increased trafficking of NMDAr into the vicinity of release sites in response to decreased activation of the existing receptors during the period of block. In contrast, when we activated presynaptic receptors with NMDA the frequency of mEPSCs greatly increased (see also Woodhall *et al.* 2001), but this was succeeded by a persistent decrease. Thus, we could postulate that mobility of the presynaptic NMDAr receptors is involved in an intermediate form of self-regulation of synaptic strength at glutamate synapses. We know that the presynaptic NMDAr are likely to be exclusively NR2B-containing (Woodhall *et al.* 2001; Chamberlain *et al.* 2008), so the level of activity at the presynaptic NR2B receptors may control an activity-dependent signal leading to accelerated or decreased mobility, and increased or decreased numbers of the same receptor close to release sites. As noted above, blockade of NMDAr with 2-AP5 *in vivo* appears to result in trafficking of NR2B subunits *out* of synaptic terminals in somatosensory cortex (Fujisawa & Aoki, 2003), but these studies were conducted immediately following the 2-AP5 application, not after recovery so it is difficult to relate these studies to ours. In the context of activity-dependent mobility, it is also of interest that hearing loss induced by cochlear ablation in young (P10) gerbils rapidly results in an increase in the number of presynaptic NR2B subunits at synapses in the superficial layers of auditory cortex (Kotak *et al.* 2005), and this could reflect increased trafficking of receptors induced by reduced afferent input.

Further implications for synaptic plasticity

Considerable attention has been focused recently on the role of trafficking of NMDAr in postsynaptic dendrites as a basis for long-term potentiation (LTP) and long-term depression (LTD) (Carroll & Zukin, 2002; Nong *et al.* 2004; van Zundert *et al.* 2004; Perez-Otano & Ehlers, 2005). Both forms of enduring plasticity have been demonstrated at layer V synapses (Yun *et al.* 2002; Solger *et al.* 2004; Craig & Commins, 2007). However, our results suggest that surface mobility of postsynoptic NMDAr is unlikely to be involved in NMDAr-dependent changes in synaptic strength at these synapses, although we cannot rule out a contribution of NMDAr exchanging between membrane and cytoplasmic stores.

There is increasing evidence that presynaptic NMDAr are involved in LTP (Humeau *et al.* 2003; Samson & Pare, 2005) and LTD (Sjöström *et al.* 2003; Bender *et al.* 2006; Corlew *et al.* 2007) at other glutamate synapses. We do not yet know whether presynaptic NMDAr can act as mediators or modulators of long-term plasticity in the EC. If they do, the ability of NMDAr to alter synaptic transmission by migration in the presynaptic membrane could well play a role in plasticity or metaplasticity and we now aim to investigate this possibility. Finally, we have previously shown that presynaptic NMDAr function is greatly enhanced in the EC in chronically epileptic animals, and that some anticonvulsants may target presynaptic NMDAr (Yang *et al.* 2006, 2007). Whether alterations in presynaptic NMDAr mobility are a factor in the action of anticonvulsant drugs and whether a pathological change in mobility may contribute to chronic epileptogenesis, will also be subjects for future investigation.

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Research Article

The Role of NMDA Receptor Subtypes in Short-Term Plasticity in the Rat Entorhinal Cortex

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We have previously shown that spontaneous release of glutamate in the entorhinal cortex (EC) is tonically facilitated via activation of presynaptic NMDA receptors (NMDAr) containing the NR2B subunit. Here we show that the same receptors mediate short-term plasticity manifested by frequency-dependent facilitation of evoked glutamate release at these synapses. Whole-cell patch-clamp recordings were made from layer V pyramidal neurones in rat EC slices. Evoked excitatory postsynaptic currents showed strong facilitation at relatively low frequencies (3 Hz) of activation. Facilitation was abolished by an NR2B-selective blocker (Ro 25-6981), but unaffected by NR2A-selective antagonists (Zn^{2+}, NVP-AAM077). In contrast, postsynaptic NMDAr-mediated responses could be reduced by subunit-selective concentrations of all three antagonists. The data suggest that NMDAr involved in presynaptic plasticity in layer V are exclusively NR1/NR2B diheteromers, whilst postsynaptically they are probably a mixture of NR1/NR2A, NR1/NR2B diheteromers and NR1/NR2A/NR2B triheteromeric receptors.

1. INTRODUCTION

A huge amount of research has been devoted to the study of the physiology, pharmacology, function, and pathology of NMDA receptors (NMDAr). This has been extensively reviewed elsewhere (e.g., [1–6]). Native NMDAr are heteromeric structures, and consist of NR1 subunits, which are obligatory, in combination with one or more of four subtypes of NR2 subunit (NR2A-D). Functional receptors are tetramers, comprising two NR1 subunits and two NR2 subunits, where the functional unit is probably an NR1/NR2 heterodimer. The functional properties of NMDAr, such as single channel conductance, the degree of voltage-dependent Mg^{2+} block, and deactivation kinetics depend on which of the four NR2 subunits is assembled in the receptor. For example, NR2A and NR2B-containing channels have a high single channel conductance (40–50 pS) whereas NR2C and NR2D are lower (15–35 pS). NR2A-containing receptors display fast decay kinetics (around 100 milliseconds), whereas NR2B and C are much slower (250 milliseconds), and NR2D slower still (4 seconds) [5, 7]. In addition to functional differences, various subunit combinations display pharmacological differences in susceptibility to antagonists and regulatory mechanisms (such as sensitivity to H^+, Zn^{2+}, polyamines).

Synaptic transmission is a highly dynamic and plastic process, modified on-demand by a myriad of instantaneous, short, intermediate, and long-term regulatory mechanisms. Much attention has been devoted to the study of the role of NMDAr in synaptic plasticity, particularly in long-term potentiation (LTP) and depression (LTD). These studies have largely focussed on NMDAr at postsynaptic sites. However, dynamic regulation of synaptic strength can also involve receptors on presynaptic terminals, which provide a powerful, synapse-delimited control of transmitter release, and the existence of presynaptic NMDAr (preNMDAr) is now firmly established. Neurochemical [8–11] and immunolocalization studies [12–15] provided early indications for preNMDAr. We provided the first clear functional demonstration of preNMDAr, showing that the competitive antagonist, 2-AP5, could reduce the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) at glutamate synapse in the rat entorhinal cortex (EC), indicating a tonic facilitatory effect of preNMDAr on glutamate release [16]. PreNMDAr

are now known to modify both glutamate and GABA release in a wide variety of locations and tissues [17–33].

Increasing attention is being paid to the role of preNMDAr as mediators of both long-term alterations in synaptic strength, and in moment-to-moment and short-term activity-dependent changes in transmitter release. For example, a role of preNMDAr in LTD has been demonstrated in cerebellum [34], visual [22, 33], and somatosensory [17] cortex. Conversely, involvement of preNMDAr in LTP has been demonstrated in amygdala [26, 32]. More intermediate forms of potentiation of glutamate [30] and GABA transmission [23], over a time scale of minutes, may also involve preNMDAr. As noted above, we found that preNMDAr are tonically activated by ambient glutamate [17, 35], providing instantaneous control over the level of glutamate release at EC synapses. Similar results have been reported for other areas [22, 27, 28, 33]. In addition, we found that preNMDAr are activated after action potential-driven synaptic release of glutamate, increasing the probability of subsequent release and allowing them to mediate short-term, frequency-dependent facilitation of glutamate transmission [16, 35].

We have also demonstrated that the tonic facilitatory effect of preNMDAr on spontaneous glutamate release is likely to be predominantly mediated by NR2B-containing NMDAr, since the increase induced by 2-AP5 was mimicked [35, 36] by relatively specific blockers of the NR2B subunit, ifenprodil [37], and Ro 25-6981 [38]. In addition, an antagonist with some specificity (albeit weak) for the NR2A subunits, NVP-AAM077 [39] had little effect. Others have also concluded that preNMDAr are likely to be predominantly NR2B-containing [27, 33, 40]. Postsynaptically, both NR2A and NR2B contribute to glutamate transmission, although there is controversy over whether diheteromeric NR1/NR2A and NR1/NR2B coexist at the postsynaptic density, or are segregated between synaptic and extrasynaptic locations, or even in a synapse-specific way [3]. The contribution of triheteromeric NR1/NR2A/NR2B receptors is also still a matter of debate [3, 41].

In the present study, we have extended our studies in the EC to examine the contribution of NR2A and NR2B receptors to short-term plasticity of glutamate transmission, by examining the effects of relatively specific blockers on the preNMDAr mediated, frequency-dependent facilitation of evoked glutamate release. In addition, we have used the same agents to determine whether postsynaptic NMDAr may differ from those on presynaptic terminals.

2. METHODS

2.1. Slice preparation

Experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act 1986, European Communities Council Directive 1986 (86/609/EEC), and the University of Bath ethical review document. Slices containing EC and hippocampus were prepared from male Wistar rats (P28–35), which were anaesthetized with an intramuscular injection of ketamine (120 mg/kg) plus xylazine (8 mg/kg) and decapitated. The brain was rapidly removed and immersed in oxygenated artificial cerebrospinal fluid (aCSF) chilled to 4°C. Slices (350–400 μm) were cut using a Vibroslice, and stored in aCSF bubbled with 95% O_2/5% CO_2, at room temperature. Following recovery for at least 1 hour, individual slices were transferred to a recording chamber mounted on the stage of a Zeiss Axioskop FS or an Olympus BX50WI microscope. The chamber was perfused (2.0 ml/min) with oxygenated aCSF (pH 7.4) at 31–33°C. The aCSF contained (in mM) NaCl (126), KCl (3), NaH_2PO_4 (1.4), $NaHCO_3$ (19), $MgSO_4$ (2), $CaCl_2$ (2), and D-glucose (10). Neurones were visualized using differential interference contrast optics and an infrared video camera.

2.2. Electrophysiological recording

Patch pipettes were pulled from borosilicate glass on a Flaming/Brown microelectrode puller. For recording spontaneous (sEPSCs) or evoked (eEPSCs) excitatory postsynaptic currents, pipettes were filled with a Cs-gluconate-based solution containing (in mM) D-Gluconate (100), HEPES (40), QX-314 (1), EGTA (0.6), NaCl (2), $MgCl_2$ (5), TEA-Cl (1), phosphocreatinine (5); ATP-Na (4), GTP-Na (0.3), MK-801 (2). Solutions were adjusted to 290 mOsmol, and to pH 7.3 with CsOH. Whole-cell voltage clamp recordings (holding potential −60 mV unless otherwise stated) were made from neurones in layer V of the medial division of the EC, using an Axopatch 200B amplifier (Molecular Devices, Calif., USA). Series resistance compensation was not employed, but access resistance (10–30 MΩ) was monitored at regular intervals throughout each recording and cells were discarded from analysis if it changed by more than ±10%. Liquid junction potential (12.3 mV) was estimated using the Junction Potential Calculator included in pClamp-8 software (Molecular Devices, Calif., USA), and compensated for in the holding potentials.

eEPSCs were elicited by electrical stimulation (bipolar pulses, 10–50 V, 0.02 millisecond duration) via a bipolar tungsten electrode placed on the surface of the slice in layer V of the lateral EC. The stimulation intensity was adjusted to give submaximal (approx. 50–60% maximum amplitude) responses.

2.3. Monitoring presynaptic NMDAr activity

In all these experiments, MK-801 (2 mM) was included in the patch pipette solution to block postsynaptic NMDAr. This allowed us to record AMPA-receptor mediated responses in isolation, and to monitor activity at preNMDAr uncontaminated by postsynaptic receptor effects. This approach was developed by us [16, 35, 42], and has been used successfully by others to block postsynaptic NMDAr in the recorded neurone [17, 27, 28, 32, 33, 40]. When whole-cell access was gained, neurones were voltage clamped at 0 mV, and synaptic stimulation was delivered at 2 Hz for 30–40 seconds to allow blockade of postsynaptic NMDAr by MK-801 dialyzed into the cell via the patch pipette solution. Membrane potential was then clamped at −60 mV and single shock stimulation delivered at low frequency (0.05 Hz) to

evoke AMPAr mediated EPSCs. At 2 or 3 minute intervals, the single shock was replaced with stimulation at 3 Hz for 10 seconds. Such stimulation results in a frequency-dependent facilitation of the AMPAr-mediated EPSC, which we have shown previously to be dependent on activation of preNMDAr [35]. We used the degree of frequency-dependent facilitation of AMPAr-mediated eEPSCs as a quantitative measure of preNMDAr activation.

2.4. Monitoring postsynaptic NMDAr activity

In these experiments, MK-801 was omitted from the patch pipette solution. When whole-cell access was gained, control eEPSCs were recorded at a holding potential of −60 mV, before addition of the AMPAr antagonist, NBQX, and the $GABA_A$r-antagonist, bicuculline to the bath perfusion. After 10–12 minutes, the holding potential was changed to +40 mV to record isolated NMDAr-mediated EPSCs as positive going currents. These were evoked at low frequency (0.05 Hz) until stable amplitudes were recorded, before addition of antagonists to the bath.

2.5. Data analysis

Data were recorded to computer hard disk using Axoscope software. Minianalysis (Synaptosoft, Decatur, Ga, USA) was used for analysis of EPSCs offline. In the studies of preNMDAr, the average peak amplitude of the 8 responses before each episode of 3 Hz stimulation was determined. During the period of 3 Hz stimulation, the amplitude of the 8 largest events was determined and normalized to the average amplitude of the preceding low-frequency events to obtain a quantitative measure of frequency-dependent facilitation in the presence and absence of antagonists. In these studies, we also analyzed AMPAr-mediated sEPSCs, by determining interevent interval (IEI), amplitude, rise (10–90%), and decay times. sEPSCs were detected automatically using a threshold-crossing algorithm. Threshold varied from neurone to neurone but was always maintained at a constant level in any given recording. At least 200 events were sampled during a continuous recording period for each neurone under each condition. Cumulative probability distributions of IEI were compared using the Kolmogorov-Smirnoff test. In experiments on postsynaptic NMDAr, responses were quantified by measuring mean peak amplitudes of at least 5 NMDAr-mediated eEPSCs evoked at low frequency at intervals throughout the study. In these studies, the vast majority of sEPSCs were blocked, as recordings were conducted in the presence of NBQX. Occasional slow sEPSCs mediated by NMDAr were recorded, their frequency was very low (2-3 per minute) and precluded meaningful analysis.

2.6. Materials

Salts used in preparation of aCSF were "Analar" grade and purchased from Merck/BDH or Fisher Scientific (Dorset, UK). All drugs were applied by bath perfusion. MK-801, NMDA, NBQX, D-2-AP5, bicuculline methiodide, and Ro 25-6981 ((αR,βS)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol hydrochloride) were obtained from Tocris (Bristol, UK). TPEN (N,N,N′, N′-Tetrakis-(2-pyridylmethyl)-Ethylenediamine) was obtained from Sigma (UK). UBP302 ((S)-1-(2-amino-2-carboxyethyl)-3-(2-carboxybenzyl) pyrimidine-2,4-dione) was a kind gift from Dr. Dave Jane, University of Bristol, and NVP-AAM077 ((R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid) was a gift from Dr. Yve Auberson at Novartis (Basel, Switzerland).

3. RESULTS

3.1. Presynaptic NMDAar

Figure 1(a) shows eEPSCs evoked in a layer V neurone at 3 Hz, with postsynaptic NMDAr blocked by internally dialyzed MK-801. The first 6 responses evoked during a train of 30 at 3 Hz are shown and demonstrate the facilitation seen at this relatively low frequency. As reported previously [35], the facilitation of the AMPAr-mediated eEPSCs was entirely dependent on presynaptic NMDAar activation, since it could be abolished by 2-AP5 ($n = 5$, Figure 1(b)). Likewise, the NMDAr channel blocker, MK-801, also abolished frequency facilitation ($n = 10$, Figure 1(b)). In some neurones, facilitation was replaced by a weak frequency-dependent depression of eEPSCs in the presence of the blockers. This can be seen as a reduction in mean amplitude of eEPSCs in the presence of the blockers (e.g., Figure 1(b)). In a further 5 neurones, we confirmed the specificity of the effect by testing the effects of GluR5 subunit specific antagonist of kainate receptors (UBP 302, 20 μM), since we have recently shown that these receptors mediate a similar short-term facilitation of glutamate transmission at 3–5 Hz in layer III of the EC (Chamberlain S.E.L and Jones R.S.G. unpublished). UBP 302 had no effect on facilitation in layer V (not shown) confirming its dependence on NMDAar. Interestingly, 2-AP5 had no effect on frequency facilitation in layer III of the EC (not shown), so although similar short-term plasticity is seen in both layers, its underlying mechanism is lamina-specific.

Since neither 2-AP5 nor MK-801 has selectivity for NR2A v NR2B subunits [5], the data do not indicate the subunit composition of NMDAr responsible for short-term frequency-facilitation. To determine the receptor involved, we have examined the effect of more specific antagonists. First, we tested the effects of Ro 25-6981. This is an allosteric inhibitor of NMDA receptors, which binds to a site on the N-terminal domain of the NR2 subunit, with a high degree of selectivity (>3000 fold) for NR2B over NR2A [38]. Figure 2(a) shows that Ro 25-6981 at 500 nM abolished the frequency facilitation of eEPSCs, again revealing a weak depression. A lower concentration (200 nM, $n = 3$) of Ro 25-6981 resulted in a mean maximal reduction in frequency-facilitation of 69 ± 7%. At these concentrations, the drug should have little or no effect on NR2A subunits [38], strongly suggesting that NR2B-containing receptors are primarily responsible for this form of short-term plasticity at layer V synapses. This would agree with previous studies that have shown the tonic facilitatory effect on spontaneous

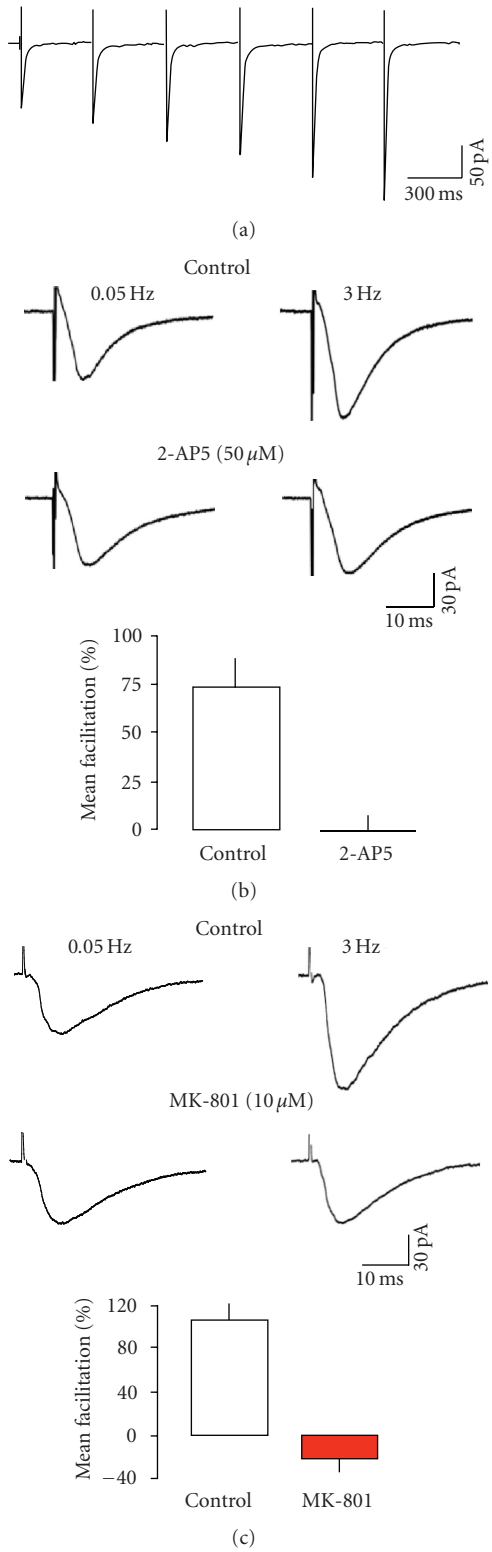

Figure 1: Short-term facilitation is mediated by presynaptic NMDA receptors. (a) First 6 responses evoked by a train of stimuli (3 Hz, 20 seconds) averaged from 3 neurones. (b) Responses ($n = 8$) were averaged at low frequency and during 3 Hz stimulation. In the presence of 2-AP5, low-frequency responses were unaltered, but facilitation was abolished. The bar graphs show the mean results from 5 neurones. (c) Similar results were seen with MK-801. Stimulation artifacts have been partially blanked for clarity.

release is likely to be NR2B-mediated [16, 35, 43]. Accordingly, Ro 25-6981 resulted in a substantial increase in IEI of sEPSCs from 277 ± 82 milliseconds (5.5 ± 1.9 Hz) to 764 ± 261 milliseconds (2.1 ± 0.7 Hz) recorded in the same neurones (cf. [36, 43]). KS analysis of cumulative probability distributions confirmed a highly significant change. There was no concurrent change in mean amplitude, rise, or decay time (not shown).

Next, we examined the effect of NVP-AAM077 in 5 neurones. This is a competitive antagonist that shows some selectivity for receptors containing the NR2A subtype. Initial reports indicated a greater than 100 fold selectivity of the compound for NR2A over NR2B [39, 44]. However, recently, it has been suggested that the selectivity is closer to 10 fold when the affinity of the two subtypes for glutamate is accounted for ([41], see also [45, 46]). Thus, at the concentration employed here (400 nM), we might expect almost complete blockade of NR2A receptors, but it is possible that substantial inhibition of NR2B would also occur [41]. Nevertheless, NVP-AAM077 did not significantly affect the frequency-dependent facilitation of eEPSCs (see Figure 2(b)). If anything, the facilitation was slightly (although not significantly) increased. These data suggest that NVP-AAM077 may have reasonable selectivity for the NR2A receptor in our preparation, but that these receptors are not involved in presynaptic short-term plasticity at layer V synapses. Further support for this was obtained from analysis of sEPSCs. The mean IEI in control was 443 ± 230 milliseconds (4.0 ± 0.9 Hz), and this decreased slightly to 377 ± 180 milliseconds (4.5 Hz) with the addition of NVP-AAM077. Likewise, there was no change in amplitude, rise, or decay times of sEPSCs (not shown).

In view of the controversy over the selectivity of NVP-AAM077, we also tested ($n = 5$) the effects of Zn^{2+}, which has been shown to discriminate between NR2A and NR2B receptors. Like Ro 25-6981 at NR2B subunits, Zn^{2+} binds to the N-terminal domain of the NR2A subunit to exert a voltage-independent inhibition with >100 fold selectivity over NR2B [47–49]. However, as with NVP-AAM077, a relatively high concentration of Zn^{2+} (300 nM) failed to alter frequency-dependent facilitation of eEPSCs (see Figure 2(c)). In addition, it had little effect on the IEI (200 ± 150 *v* 298 ± 170 milliseconds, see Figure 2(d)), amplitude (17.7 ± 3.4 *v* 15.4 ± 2.2 pA), rise (1.9 ± 0.3 *v* 2.1 ± 0.4 milliseconds), or decay times (24.6 ± 1.6 *v* 27.3 ± 1.3 milliseconds) of sEPSCs (cf. [43]). Thus, the data from both NVP-AAM077 and Zn^{2+} studies militate strongly against a role for NR2A receptors in presynaptic frequency-dependent facilitation in layer V of the EC. The ability of Ro 25-6981 to block facilitation strongly indicates that presynaptic plasticity at these synapses is dependent only on NR2B-containing receptors.

A recent paper [50] suggested that activation of postsynaptic NR2B-containing receptors at a similar frequency (3.3 Hz) to that employed by us to elicit frequency-dependent facilitation induced a long-term depression of the NMDAr-mediated currents themselves (primarily by decreasing fractional Ca^{2+} currents carried by the receptors). We were interested to see if the repetitive activation of the

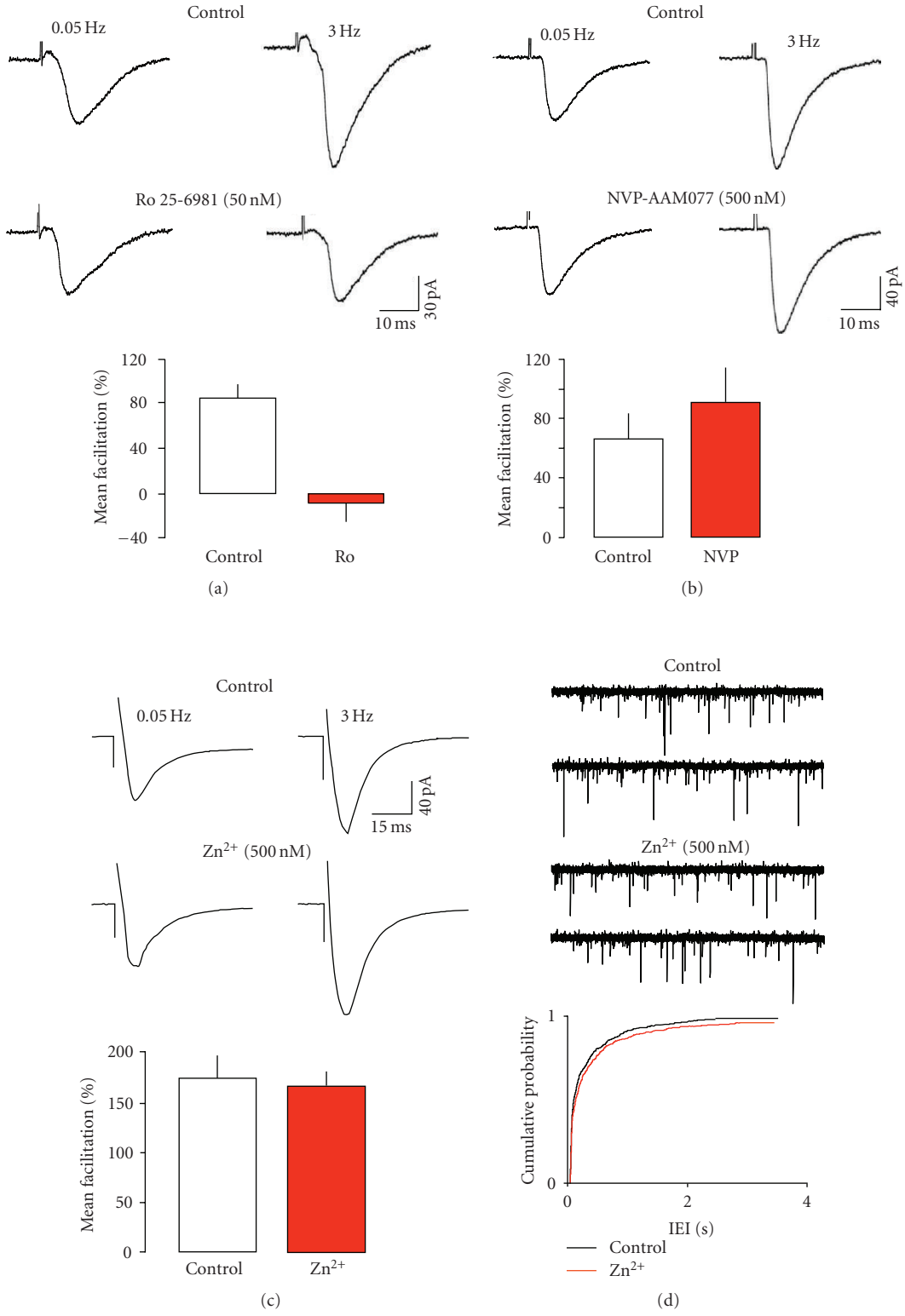

Figure 2: Effects of subunit selective antagonists. (a) Ro 25-6981 abolished frequency-dependent facilitation. In contrast, neither NVP-AAM077 (b) nor Zn^{2+} (c) had any significant effect. (d) Zn^{2+} also had little effect on sEPSCs. The records show consecutive sweeps of baseline recording of sEPSCs and in the presence of Zn^{2+}. The cumulative probability plots show pooled data from 6 neurones, with 200 events from each neurone in the presence and absence of the blocker. There was a small shift to the right in the presence of Zn^{2+}, but this failed to reach significance (KS test).

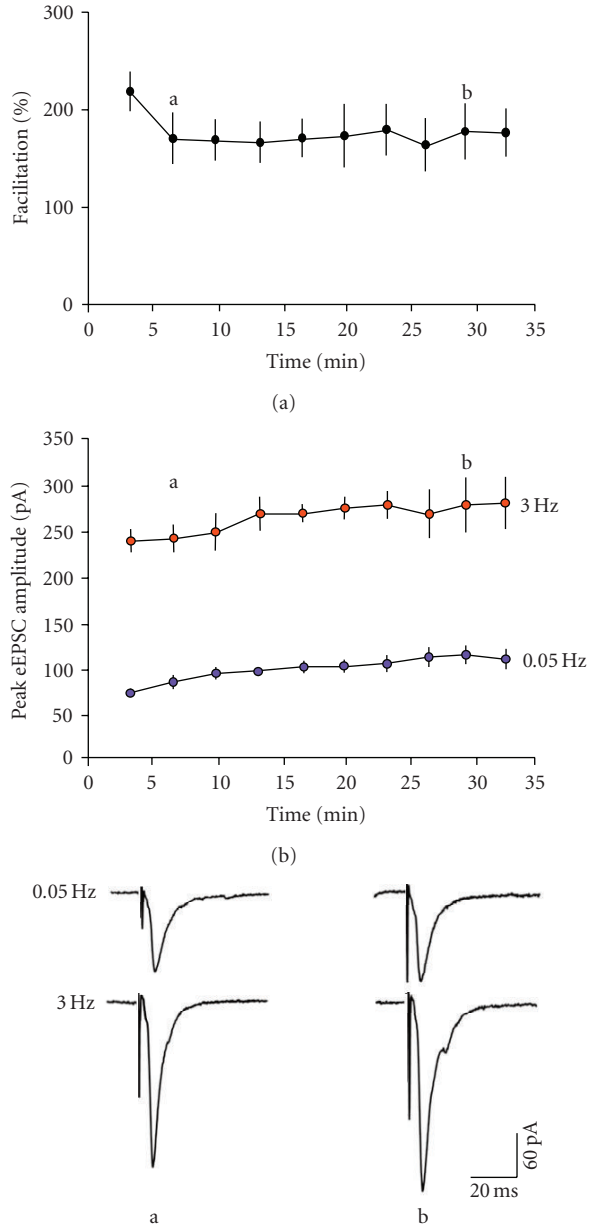

Figure 3: Progressive changes associated with repeated episodes of stimulation at 3 Hz in the absence of NMDAr blockers. Each point is the degree of facilitation recorded during a 30-second period of stimulation and is the average from 5 neurones. (a) After an initial decline in the degree of facilitation, it remained stable throughout the subsequent 30 minutes of recording. (b) Mean amplitude of responses recorded at low and high frequency used to assess the facilitation in the neurones shown in (a). There was a progressive, albeit small increase in amplitude of responses in both cases. Representative records from one neurone, sampled at the times indicated, are shown below.

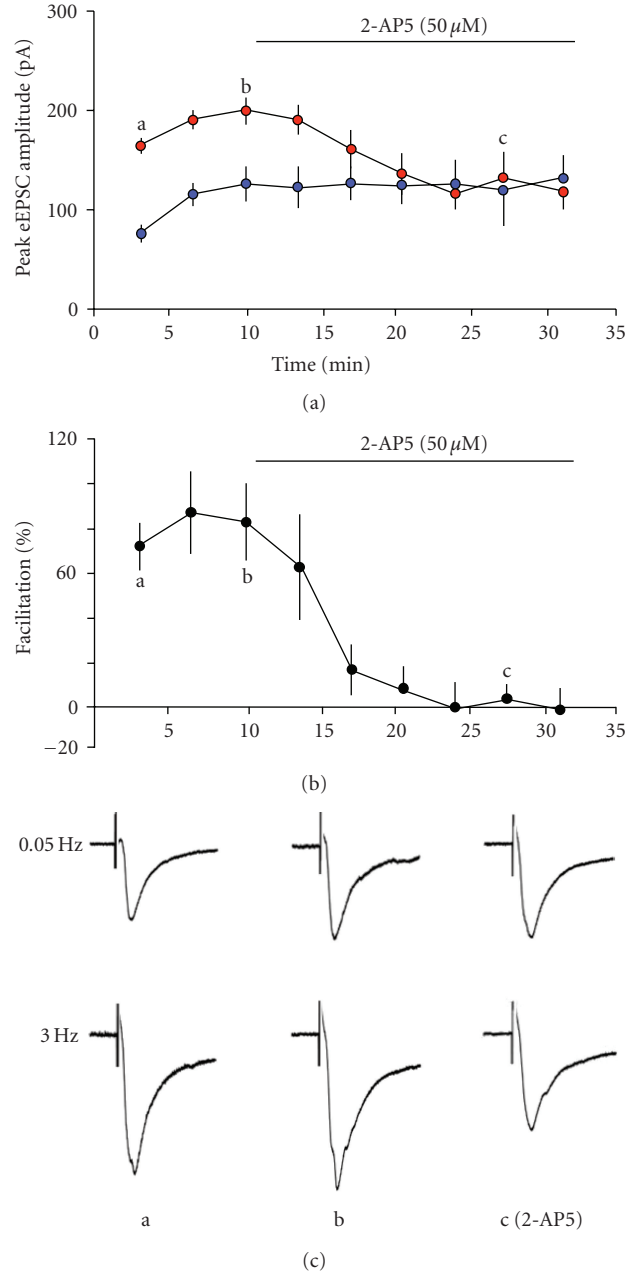

Figure 4: Time course of the effect of 2-AP5 on eEPSC amplitude and facilitation. (a) The progressive increase in both low- and high-frequency responses was prevented by the addition of 2-AP5 (n = 5 neurones). The responses at high frequency were progressively reduced to control levels, in parallel with the degree of facilitation (b). (c) Representative responses recorded in one neurone at the times indicated.

presynaptic NR2B-containing receptors would induce any decrement in frequency facilitation at layer V synapses. In 5 neurones, we induced facilitation of eEPSCs and monitored the degree of facilitation but without the addition of any blockers. Overall there was an initial decrease in the degree of facilitation of AMPAr-mediated eEPSCs from the first to second episode, but thereafter it was remarkably consistent (see Figure 3(a)). However, when we looked at absolute amplitude of eEPSCs, there was a small, but consistent, increase over the course of the studies. This applied to events evoked at both low and high frequencies (see Figure 3(b)). We also examined the time course of these changes in the neurones tested with 2-AP5 (see Figure 4). The antagonist appeared to prevent the progressive increase in amplitude

of the low-frequency events at the same time as blocking the frequency-dependent facilitation. This limited protocol may suggest the short-term frequency-dependent facilitation could underlie a longer-term enhancement of glutamate transmission. As the postsynaptic NMDAr were already blocked (by internal MK-801), this is likely to involve the presynaptic, NR2B-containing receptors.

3.2. Postsynaptic NMDAr

We now wished to determine the contribution of NR2A/B subunits to NMDAr at postsynaptic sites in layer V of the EC, so we tested the same antagonists used in the presynaptic experiments for effects on isolated NMDAr-mediated eEPSCs. As expected, the nonspecific blockers 2-AP5 ($n = 5$) and MK-801 ($n = 9$) both abolished the slow eEPSCs recorded at +40 mV in the presence of NBQX and bicuculline (not shown). Ro 25-6981 ($n = 5$) also elicited a concentration dependent reduction in postsynaptic NMDAr responses at concentrations that would be expected to retain selectivity for NR2B-containing receptors (see Figure 5(a)). The slow eEPSCs were essentially abolished by Ro 25-6981 at 500 nM. This suggests that NR1/NR2B receptors dominate at postsynaptic sites as they do presynaptically. However, when we tested NVP-AAM077 ($n = 6$), we again found a concentration-related reduction in postsynaptic responses with around 80% inhibition at 500 nM (see Figure 5(b)). Comparison with the data of Neyton and Paoletti [41] suggests that the effect of NVP-AAM077 could be explained by blockade of both NR2B and NR2A receptors since 500 nM was sufficient to abolish NR2A responses in oocytes, but also to exert around 60% block of NR2B. However, this is at odds with its failure to alter preNMDAr-dependent facilitation, which is clearly an NR2B-mediated response. Studies with Zn^{2+} ($n = 6$) failed to substantially clarify the situation. The divalent cation also elicited a concentration-dependent reduction in slow eEPSCs (see Figure 5(c)). The concentrations employed exert around an 80% voltage-independent block of NR2A receptors expressed in oocytes, but retain a considerable degree of selectivity with regard to block of NR2B receptors [47, 49]. These data do suggest a role for NR2A receptors at postsynaptic sites, but it is puzzling that Ro 25-6981 essentially also abolished NMDAr EPSC, when it would be expected to have little effect on NR2A receptors.

We performed two more sets of experiments to look at this question further. In 5 neurones, we first perfused a low concentration of Ro 25-6981 (200 nM), to partially block the NMDAr EPSC. We then added a low concentration of Zn^{2+} (100 nM). In these neurones, Ro 25-6981 resulted in inhibition of around 45%, and with the addition of Zn^{2+} there was a further reduction to around 90–100%, which clearly indicates a role of both NR2A and NR2B in mediating the postsynaptic response (see Figure 5(d)). Finally, there is evidence that under control conditions, NR2A-containing receptors may be substantially blocked by Zn^{2+}, present in the ACSF as a result of contamination of other salts used in its preparation [47]. Although addition of Zn^{2+} clearly reduced slow eEPSCs in our experiments, we also examined whether there was significant blockade of the NR2A receptor in control recordings by testing the effect of the Zn^{2+}-chelator, TPEN (2 μM), in 3 neurones. This had no effect on the mean amplitude of NMDAr eEPSCs (125.3 ± 25.1 *v* 111.9 ± 26.1 pA) suggesting that our results with antagonists were unlikely to be confounded by Zn^{2+}-contamination.

Finally, as noted above, relatively low frequency, repetitive activation of NR2B receptors has been shown to induce a depression of postsynaptic NMDA responses per se [50]. In 7 neurones, we determined the effects of a brief period of repetitive stimulation (3 Hz, 40 seconds) on postsynaptic NMDAr eEPSCs in 5 neurones. Overall, during the repetitive stimulation there was a small (15%), progressive decrease in the first 10–15 seconds, and thereafter the amplitude reached a plateau (see Figure 6(a)). We then recorded NMDAr eEPSCs at low frequency (0.05 Hz) over the subsequent 30 minutes. There was an initial period (5 minutes) where responses appeared to be slightly depressed and thereafter a recovery followed by a slight increase before recovery to control levels (see Figure 6(b)). However, apart from a brief period around 20 minutes there was no significant difference compared to control.

4. DISCUSSION

We originally demonstrated that the presynaptic NMDAar mediating facilitation of glutamate release in the EC was likely to be predominantly NR2B-containing, as the frequency of sEPSCs was decreased by the N2B antagonist, ifenprodil [35]. Other work supports the conclusion that preNMDAr that facilitate spontaneous glutamate release at cortical synapses are primarily NR2B-containing. We found that Ro 25-6981 but not NVP-AAM077 or Zn^{2+} reduced sEPSC frequency ([36], present study), and similar results with Ro 25-6981 and Zn^{2+} were reported for synapses in layer II/III of the visual cortex [28]. Jourdain et al. [27] reported that presynaptic NR2B receptors were responsible for the increase in mEPSC frequency in dentate granule neurones seen after stimulation of glutamate release from adjacent astrocytes, as it was blocked by ifenprodil. We now show that the same receptor is likely to mediate short-term plasticity of evoked glutamate release in layer V of the EC. Thus, the facilitation of eEPSCs at the relatively low frequency of 3 Hz was blocked by Ro 25-6981. The lack of effect of NVP-AAM077 and Zn^{2+} suggests that NR2A receptors do not contribute to facilitation of either spontaneous or evoked glutamate release at EC synapses. We cannot rule out a role of NR2A receptors at higher frequencies, although Sjöström et al. [33] have reported that frequency facilitation at 30 Hz at layer V synapses in visual cortex is greatly reduced by ifenprodil, suggesting that NR2B dominate at other presynaptic sites as well.

It is somewhat surprising that only presynaptic NR2B receptors appear to modulate release. Postembedding immunolabeling studies have shown the presence of NR1 subunits in presynaptic terminals in cortex and hippocampus [12–14, 51–53]. Whilst a host of studies have demonstrated NR2B subunits at presynaptic locations [15, 51, 54–59], similar studies have also indicated the presence of NR2A

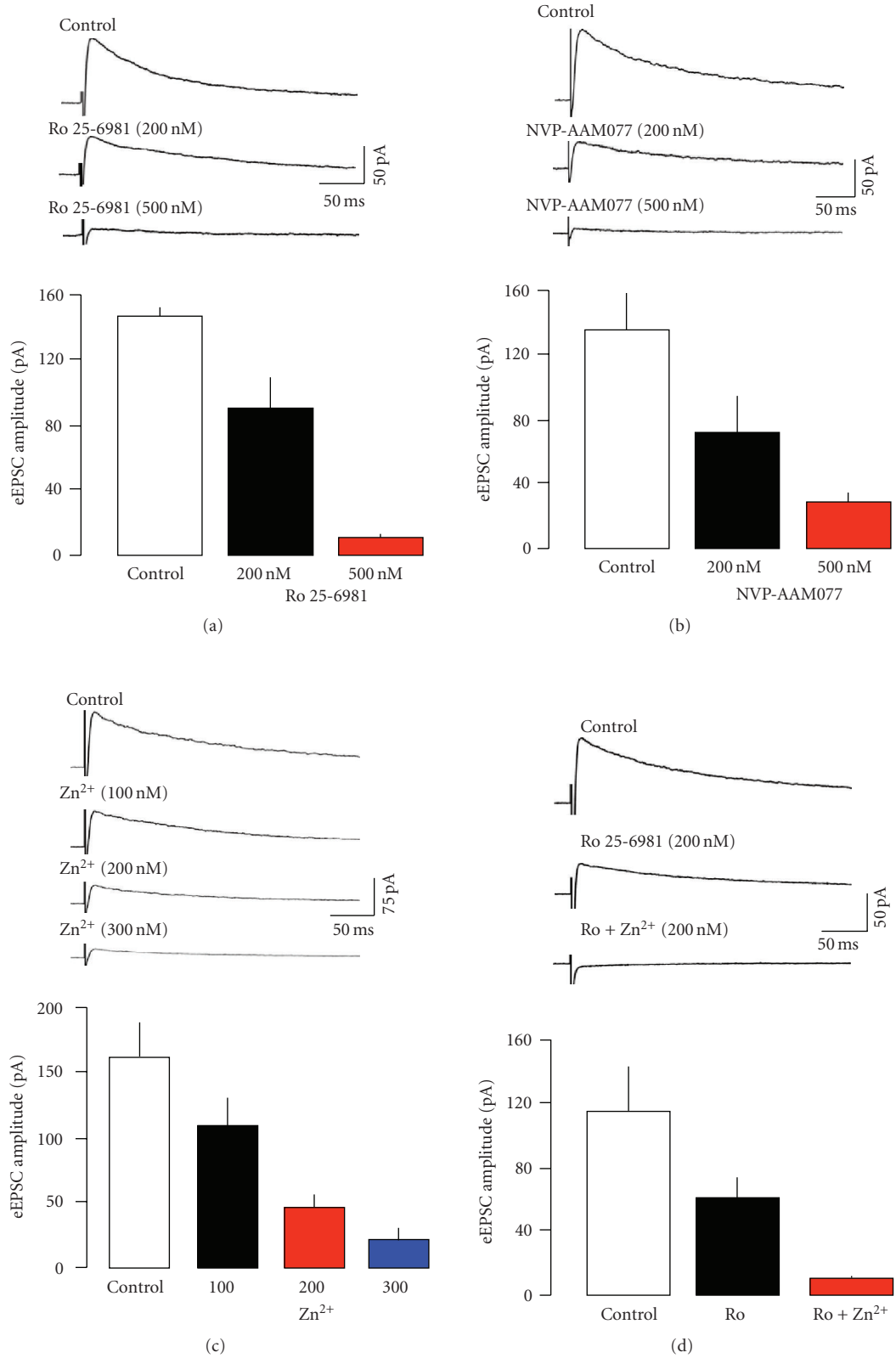

Figure 5: Effect of subunit selective antagonists on postsynaptic NMDAr-mediated eEPSCs. Slow eEPSCs were recorded at +40 mV in the presence of NBQX and bicuculline. Each response is the average of at least 8 events. (a) The NR2B antagonist, Ro 25-691, induced a concentration-dependent reduction in slow eEPSCs. They were essentially abolished at the higher concentration. (b) and (c) show that NR2A selective blockers induced a very similar blockade of slow EPSCs. (d) A combination of NR2A and NR2B antagonists also abolished slow EPSCs.

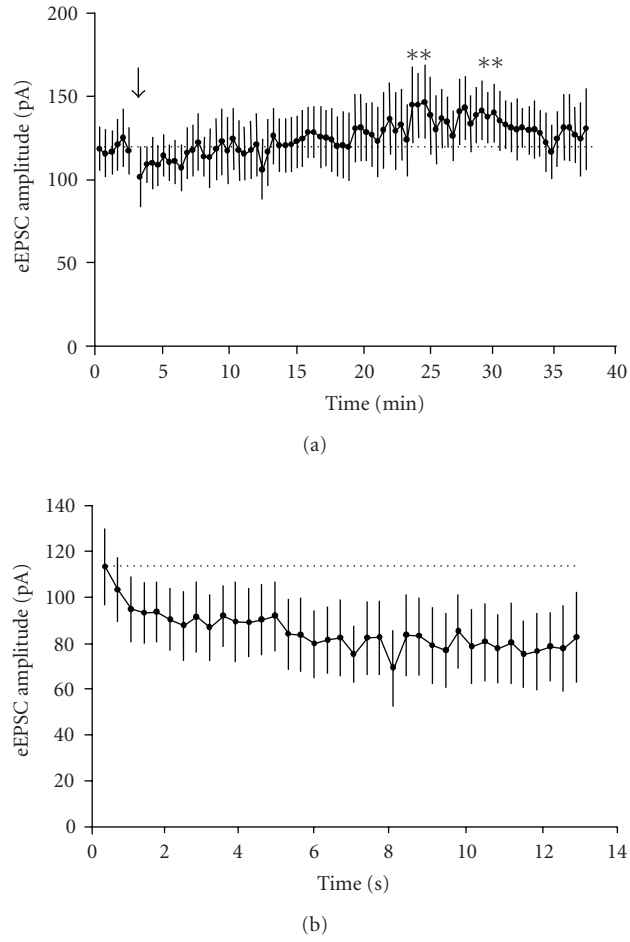

Figure 6: Changes in slow eEPSC amplitudes during and after repetitive stimulation at 3 Hz for 30 seconds. (a) shows the average response amplitudes at low frequency (0.05 Hz) recorded during 35 minutes stimulation in 7 neurones. During the period indicated by the arrow, stimulation was increased to 3 Hz for 30 seconds and the average response amplitudes (first 37 only for clarity) recorded during this period are shown in (b). The only significant differences compared to the mean control value are indicated by the asterisks in (a).

subunits [51, 52, 60–62] although, to date, there are no similar studies specifically related to the EC.

The presence of all three subunits suggests that both NR1/NR2A and NR1/NR2B diheteromeric receptors and possibly also NR1/NR2A/NR2B triheteromers could be expressed in cortical presynaptic terminals, and this may well be the case. However, it is clear from the pharmacological experiments presented here and elsewhere, that NR1/NR2B receptors are predominantly responsible for short-term NMDAr-mediated facilitation of glutamate release (but see, [63]). The properties of NR2B subunits differ from NR2A, in a way that may make them more suited to the task of presynaptic facilitation (see [6, 7, 64–66]). NR2B subunits have a higher affinity for both glutamate and glycine, and show less desensitization. The two subunits confer similar single channel conductance to diheteromeric receptors (around 50 pS), but they have very different deactivation kinetics, with NR1/NR2A receptors having decay time constants of 50–100 milliseconds, and NR1/NR2B receptors in the order of 200–400 milliseconds. Both are Ca^{2+}-permeable, but NR2B receptors exhibit a higher fractional Ca^{2+}-current than NR2A (see [66, 67]). Both subunits also display Ca^{2+}-dependent inactivation, but this is more pronounced for NR2A. The presence of NR2B subunits results in prolonged EPSPs compared to those seen when NR2A subunits dominate (see [3, 7, 66]). Thus, it seems likely that activation of presynaptic NR2B-containing receptors would mediate a slowly deactivating opening of the NMDAr channel and a greater Ca^{2+}-influx into the presynaptic terminals than any influx mediated by NR2A receptors. Ca^{2+}-influx via the NMDAr is responsible for instantaneous control of spontaneous glutamate release [35]. With a deactivation time of around 300 milliseconds, repetitive activation of NR1/NR2B receptors would readily result in temporal summation of presynaptic Ca^{2+}-entry leading to the short-term facilitation at even relatively low-frequency stimulations seen here and previously [35].

It is interesting to speculate on a physiological or pathological role for short-term plasticity mediated by preNMDAr. State-dependent rhythms and oscillatory activity at various frequencies occur in the networks of the EC including ripples and sharp waves (>100 Hz), gamma (30–80 Hz), theta (4–8 Hz), and slow waves (0.1–0.5 Hz) [68–71], and these may be involved in mnemonic processing in temporal lobe structures. There is a consensus that theta oscillations are intimately involved in declarative memory and spatial navigation (see [72–74]), and it is possible that information encoding involved in these processes is reliant on an increase in entorhinal-hippocampal delta/theta coherence [73]. The facilitation of glutamate transmission mediated by preNMDAr that we describe is readily elicited at frequencies in the low theta range. Thus, we could speculate that these receptors may be involved in the generation of theta activity in the EC, and the proposed role of this activity in short-term memory and coding of spatial information (e.g., [72, 74]).

At a pathological level, it is noteworthy that, oscillations at delta (1-2 Hz) and theta frequency may be associated with epilepsy. In patients with temporal lobe epilepsy, there is a generalized increase in EEG activity in the delta/theta range, and the most common pattern of discharges after the initiation of ictal events is a rhythmic delta/theta activity (e.g., [75, 76]). Also, in rats made chronically epileptic following kainic acid injection, epileptiform events in superficial layers of the EC were sometimes followed by spontaneous theta oscillations in layer V [77]. We recently showed that preNMDAr function declines in adulthood, but is markedly enhanced in age-matched, chronically epileptic rats [36] and there is evidence for a similar increased function in human temporal lobe epilepsy [78]. We could speculate that this increased preNMDAr function could result in enhanced generation of delta/theta activity in epileptic conditions. Of further interest in this regard is the observation that increased delta/theta EEG activity (albeit in patients with generalized absence/myoclonic seizures) is normalized by the anticonvulsant drugs, valproate, and lamotrigine [79–81].

We have also shown that at least one anticonvulsant drug (felbamate) can block the preNMDAr [42]. This raises the possibility that some anticonvulsants could alter delta/theta oscillations by targeting preNMDAr.

Whatever the function of short-term plasticity, and the involvement of preNMDAr in it, there is increasing evidence that these receptors may also contribute to longer term forms of plasticity, apparently mediating both LTD [17, 22, 33, 34] and LTP [26, 32] at a variety of synapses. In at least one case, LTD appears to be mediated by NR2B-containing receptors [33], so both short- and long-term plasticity of glutamate transmission could involve Ca^{2+}-influx via presynaptic NR2B receptors. We have also shown recently that preNMDAr are rapidly mobile and can diffuse between locations near release sites and more distal locations in the terminal membrane [82]. Trafficking of receptors in the presynaptic membrane appears to be influenced by ongoing activity levels, and exerts an intermediate (over 10 seconds of minutes) form of plasticity. Thus, presynaptic NR2B receptors may be heavily involved in both plasticity and metaplasticity at glutamate synapses in EC and other cortical synapses.

In the present study, we also present evidence for differences in pre- and postsynaptic NMDAr at layer V synapses. Whilst preNMDAr-mediated effects are exclusively dependent on NR1/NR2B-containing diheteromers, both NR2B and NR2A appear to contribute to postsynaptic responses. However, the relative contributions of the two subunits are not clear. The ability of low concentrations of both Zn^{2+} and Ro 25-6981 to reduce postsynaptic NMDAr responses could suggest that they are dependent on a mix of NR1/NR2A and NR1/NR2B diheteromeric receptors. However, concentrations of either blocker, that should largely retain selectivity at the respective subtypes, were able to almost abolish postsynaptic responses. This could suggest that the postsynaptic receptors could be largely triheteromeric NR1/NR2A/NR2B receptors. Although triheteromeric receptors do exhibit high affinity for both NR2A and NR2B selective blockers, it seems likely that they exhibit a reduced maximal inhibitory effect to either, and that maximal blockade requires occupation of both sites [83]. This does not fit well with our finding that combined application of low concentrations of Zn^{2+} and Ro 25-6981 could also abolish postsynaptic responses, which would better support a mediation by a mix of NR1/NR2A and NR1/NR2B diheteromeric receptors. It should also be noted that the ability of NMDA antagonists to block the receptors is not just dependent on the NR2 subunit present, but is also modified by which splice variant of the NR1 subunit with which it combines [47, 49]. We do not know which NR1 subunit(s) may be present in the EC. Thus, overall it is difficult to define exactly what the postsynaptic receptor population, but the most likely scenario is a mix of NR1/NR2A, NR1/NR2B, and NR1/NR2A/NR2B receptors.

A number of studies have suggested that NR1/NR2A, NR1/NR2B, and NR1/NR2A/NR2B receptors may contribute to postsynaptic responses at other cortical synapses [84–86]. There is support also for synapse-specific segregation of NR2A and NR2B-containing receptors (e.g., [87, 88]) and spatial segregation between subsynaptic and extrasynaptic sites (e.g., [86]). The controversy over whether subunit composition and spatial location are linked, and the difficulties in defining the role of triheteromeric receptors has been well reviewed recently [3]. We cannot make any firm conclusions regarding these aspects in the EC, but our data do suggest that postsynaptic NR1/NR2A, NR1/NR2B, and NR1/NR2A/NR2B receptors all contribute to postsynaptic responses at glutamate synapses in layer V of the EC, in contrast to presynaptic sites where NR1/NR2B receptors may have exclusive control. Increasing numbers of studies have documented LTP and LTD at synapses in the EC [89–95]. The EC is clearly a pivotal site in learning and memory functions resident in the temporal lobe. We have shown that preNMDAr mediate short-term forms of plasticity in the EC. In experiments employing a limited protocol of repetitive activation, we found that this short-term plasticity may lead to longer-term plasticity (either pre- or postsynaptically), and the aim now is to examine in detail the relationship between short-term effects and long-term plasticity and metaplasticity at these synapses.

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